Regulation of Gene Expression in the Immature Sertoli cell: A role for Oestrogens ?

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Contents

Declaration

The experiments described in this thesis are the unaided work of the author except where acknowledgement is made by reference. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Miriam Claire Fenelon
September, 2000

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Abstract

Testicular development is a tightly regulated and complex process in which Sertoli cell functional maturation plays a pivotal role. Reported increases in the incidence of male reproductive abnormalities in humans and other wildlife have been linked to increased levels of oestrogen and oestrogen-like chemicals in the environment. The function of oestrogen in male reproduction in particular during testicular development and maturation has been investigated. Oestrogen administration during postnatal testis development indirectly modifies Sertoli cell maturation. The recent discovery of oestrogen receptor-beta (ERβ) expression in Sertoli cells has provided a direct route for oestrogenic action in Sertoli cells. The aims of the present studies were firstly to examine the ontogeny of gene expression during normal prepubertal development of the rat testis at both mRNA and protein levels focusing on expression patterns of novel markers of Sertoli cell functional maturation. Secondly Sertoli cell gene expression patterns were investigated following exposure to oestrogen or testosterone in vivo or in vitro.

In the first part of the thesis changes in the temporal and spatial expression of Sertoli cell mRNA and proteins were investigated in the postnatal rat testis. Novel results regarding ERβ expression during this time of development were reported. In addition patterns of Musashi-1, GATA-1 and GATA-4 Sertoli cell expression were described including new information demonstrating changes in Musashi-1 and GATA-1 protein localisation in the Sertoli cell during the transition from proliferating cells to mature differentiated Sertoli cells. Artificially elevated levels of oestrogen in the neonatal testis delayed Sertoli cell maturation and subsequently delayed expression of GATA-1 protein demonstrating the restriction of GATA-1 protein expression to non-proliferating Sertoli cells which has not previously been described. A primary rat Sertoli cell culture system was established in which Sertoli cells retained hormonal sensitivity consistent with the expression of androgen receptor and ERβ proteins. New preliminary data describing upregulation of ERβ protein levels by oestrogen in cultured rat Sertoli cells are reported. Steroid regulation of

GATA-4 expression in cultured rat Sertoli cells was not detected in the present study at the level of either mRNA or protein. Problems obtaining sufficient numbers of Sertoli cells in culture during this investigation were overcome by employing an immortalised mouse Sertoli cell line from the H-2K^b-tsA58 transgenic mouse and confirmation of results obtained using primary cultures of rat Sertoli cells was carried out.

In conclusion, the studies outlined in this thesis have described expression patterns of markers of Sertoli cell functional maturation and preliminary data regarding their steroid regulation. Such new data can be employed in future studies to further evaluate direct effects of oestrogen and oestrogen-like chemicals on Sertoli cell behaviour in the postnatal rat testis with a view to elucidating the mechanisms involved in modification of testicular function by oestrogens.

Abbreviations

APS	Ammonium persulphate
ATP	Adenosine triphosphate
Bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CPM	Counts per minute
CTP	Cytosine triphosphate
ddH_20	Double distilled water
DAB	3,3'-diaminobenzidine
DES	Diethylstilbestrol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EB	oestradiol benzoate
EDS	ethane dimethane sulphonate
EDTA	Ethylenediaminetetraacetic acid
EE	ethinyl oestradiol
F	Forskolin
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
IgG	Immunoglobulin G
IPTG	Isopropyl β-D thiogalactopyranoside
Kb	Kilobases
kDa	Kilodaltons
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SGP-1	Sulphated glycoprotein-1
StAR	Steroidogenic acute regulatory protein
STF	Seminiferous tubule fluid
T	Testosterone
Taq	Thermus aquaticus
TBS	Tris buffered saline
TRIS	Trizma base
X-GAL	5-Bromo-4-chloro-3-indoyl β-D-galacto-pyranoside

Chapter 1.

Review of the Literature

1.1. Introduction

The impact of endogenous oestrogens, or exogenous ligands with oestrogenic activity, on the formation of the male reproductive system, and on reproductive function in adulthood, remain the subject of intensive research activity and debate (see reviews by (Sharpe, 1998; Sharpe *et al.*, 1998; Toppari *et al.*, 1996). Testosterone was always viewed as the 'male' hormone and conversely oestrogen was seen as the 'female' hormone a distinction that now appears simplistic. In males oestrogens are synthesised from androgens by the aromatase enzyme both within the brain and testes and levels within the male reproductive tract are generally higher than in the general circulation (reviewed in (Hess *et al.*, 1997). Oestrogen action is mediated via high affinity receptors which are shifted to a transcriptionally active state after ligand binding. Two forms of oestrogen receptor commonly known as alpha (ERα, ((Green *et al.*, 1986) and beta (ERβ, (Kuiper *et al.*, 1996) have been cloned and although they exhibit significant sequence homology within both their DNA and ligand binding domains (reviewed in (Saunders *et al.*, 1998) they are encoded on different chromosomes (Enmark *et al.*, 1997). Within rat testes expression of both ER isoforms has been demonstrated (Dorrington and Khan, 1993; Fisher *et al.*, 1997; Saunders *et al.*, 1998; van Pelt *et al.*, 1999).

Postnatal development of the testis is a highly complex and tightly regulated process involving a number of paracrine interactions. Sertoli cells play a pivotal role in these processes. In the postnatal rat testis, modification of Sertoli cell differentiation causes a delay in the onset spermatogenesis (De Franca *et al.*, 1995) and prevents the differentiation of mature Leydig cells (Papadopoulos *et al.*, 1987). Oestrogens have been proposed to have both direct, and indirect effects on functional maturation of Sertoli cells (Sharpe *et al.*, 1998; Sharpe *et al.*, 1995; Sharpe *et al.*, 2000) and neonatal treatment with oestrogens in vivo has recently been shown to affect the numbers of spermatozoa produced in adult rats (Sharpe *et al.*, 1995).

At the time that these studies were initiated it had been proposed that oestrogens played an essential role in male fertility (Eddy *et al.*, 1996) however exposure to elevated levels of oestrogen or chemicals with oestrogen-like activity during fetal or neonatal life had been shown to alter gene expression within the testis (Majdic *et al.*, 1997; Majdic *et al.*, 1995). The aim of the work described in this thesis was to increase our knowledge of the action(s) of oestrogen(s) in the postnatal testis focusing in particular on the proposal that oestrogens can have direct effects on Sertoli cell differentiation. To provide a background to these studies this chapter will firstly, briefly review the structural organisation of the mature testis. Postnatal development of the testis in the rat is reviewed in detail with particular reference to Sertoli cell maturation and the factors involved in this process. The final section reviews evidence and hypotheses surrounding effects of oestrogen on male testis development.

1.2. Organisation of the Adult Testis

The testis is a complex organ that is divided into two main compartments, the seminiferous tubule and the interstitial space. The seminiferous tubules contain Sertoli cells and germ cells. In adulthood the Sertoli cells provide both structural and metabolic support to the germ cells as they differentiate into mature spermatozoa and are released into the tubule lumen. Peritubular myoid cells surround the circumference of the tubule and provide support and contractile activity. The interstitial space is located between seminiferous tubules and is composed of Leydig cells, macrophages, blood vessels, lymphatics, fibroblasts, nerve fibres and fibrinonectin rich connective tissue. Leydig cells synthesise and secrete testosterone which is transported to the rest of the body via the adjacent vasculature. In the mature testis, the seminiferous tubules produce mature sperm which is in part dependent on the production of testosterone by the Leydig cells.

1.2.1. Seminiferous Epithelium

The seminiferous tubule consists of a seminiferous epithelium, basement membrane and peritubular cells (see Figure 1.1). The seminiferous epithelium is separated into two compartments by the inter-Sertoli cell junctions. Firstly,

the basal compartment between the inter-Sertoli cell junctions and the basement membrane which contains spermatogonia and secondly, the adluminal compartment above the tight junctions that contains all other germ cell types. Germ cells are arranged in a highly organised manner in close contact with Sertoli cells. In the seminiferous epithelium of the rat for example there is a fixed complement of 4-5 germ cell types, the associations of which have been divided into fourteen separate stages which make up the spermatogenic cycle (Leblond and Clermont, 1952). In humans the spermatogenic cycle comprises of six recognisable stages (see Figure 1.2). In the human, up to three different stages can be observed in a single cross section and the arrangement of stages appears to be helical (Schulze and Rehder., 1984). In contrast, in the rat and most other species the asegmental arrangement of stages along the epithelium results in a single stage observed per cross section (reviewed in (Sharpe, 1994).

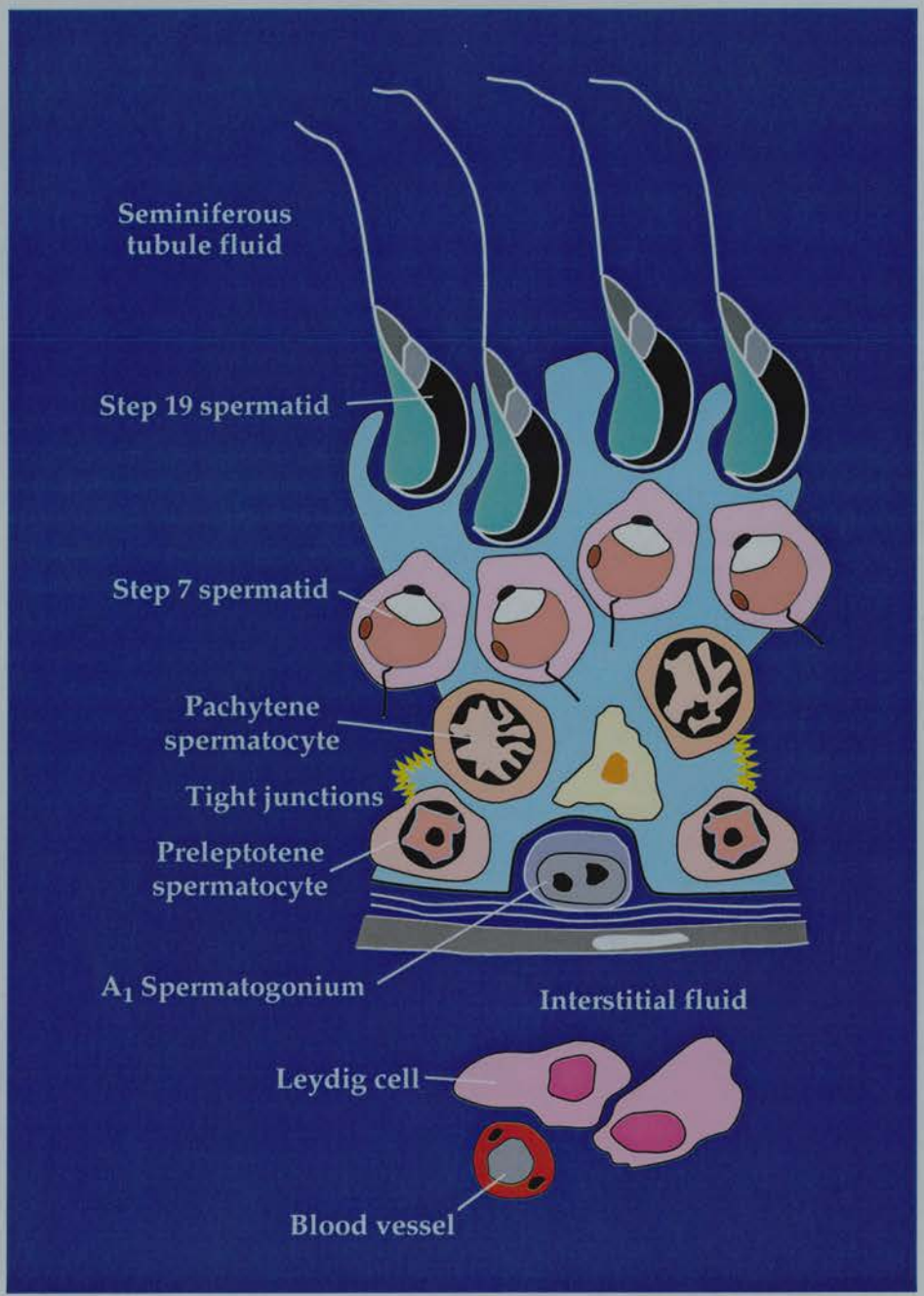

Figure 1.1: A schematic illustration of a Sertoli cell and its associated germ cells at stage VII of the spermatogenic cycle. Figure shows the cellular associations of a seminiferous tubule at stage VII of the spermatogenic cycle and the interstitial space with which it is in contact. (Adapted from (Sharpe, 1994).

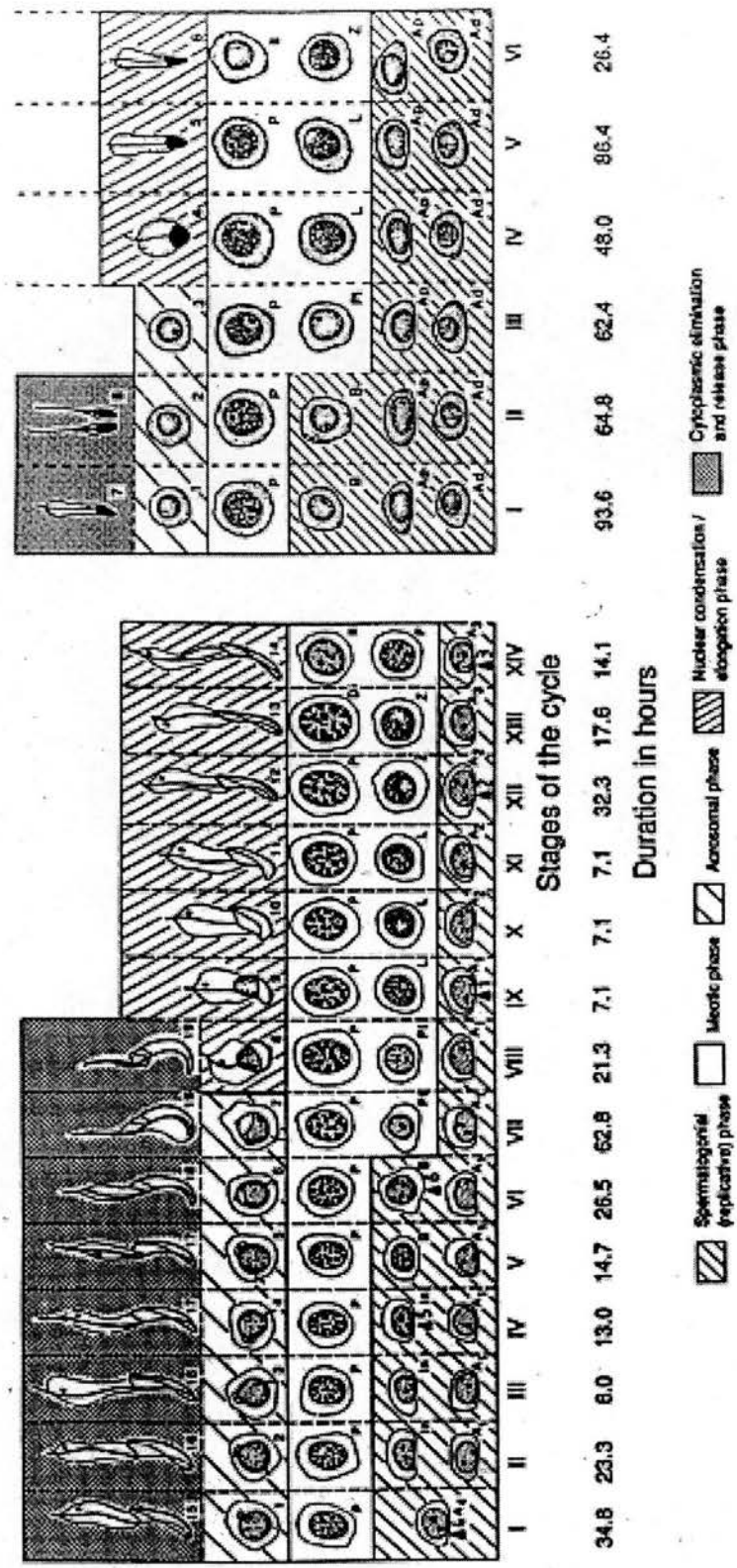

Figure 1.2: The spermatogenic cycles of the rat and human. The figure shows the different stages of the spermatogenic cycle in the rat (14 stages) and in the human (6 stages). The germ cells associated with Sertoli cells at each stage of the cycle are shown A_1-A_4, In and B = A, intermediate and B type spermatogonia; Pl, L, Z and P = preleptotene, leptotene, zygotene and pachytene spermatocytes; 1-19 = round and elongating spermatids. (Adapted from (Sharpe, 1994).

1.2.1.1. Spermatogenesis

Spermatogenesis is the process whereby a diploid type A spermatogonium undergoes a series of cellular events to become a haploid spermatozoa. In the rat each germ cell passes through the fourteen stages of the cycle 4.5 times prior to its release into the seminiferous lumen as a mature spermatozoon whilst in the human the cycle is repeated 5.5 times (see figure 1.2). The whole process of germ cell development, from the mitosis of spermatogonial stem cells to the release of spermatids takes 56 days in the rat (Clermont and Harvey, 1965) and approximately 70 days in humans.

The process of spermatogenesis can be considered as falling into three main phases; firstly, proliferation of spermatogonia, secondly, meiosis to produce haploid round spermatids and finally, spermiogenesis where round spermatids differentiate into fully mature spermatozoa. During the first phase, a spermatogonial stem cell divides into two cells, one enters the spermatogenic process and the other remains a stem cell. In the rat the stem cell entering spermatogenesis (A_0 spermatogonia) undergoes six further mitotic divisions at specific time points giving rise to A_1, A_2, A_3, A_4 and B type spermatogonia (Clermont, 1962). The progeny of each division remain connected by cytoplasmic bridges throughout proliferation and differentiation into spermatozoa (Dym and Fawcett, 1971). In man the type A spermatogonia do not undergo as many mitotic divisions as in the rat and this is believed to partly explain the reduced efficiency of spermatogenesis observed in humans (Paniagua *et al.*, 1987). B type spermatogonia undergo a final mitotic division then enter a lengthy meiotic phase as primary spermatocytes differentiating through preleptotene, leptotene, zygotene, pachytene and diplotene phases, consecutively. This is followed by two meiotic divisions which results in the formation of four haploid spermatids for each original A_0 spermatogonia, assuming no germ cell degeneration has occurred (Monesi *et al.*, 1978).

The third phase, spermiogenesis, involves the structural remodelling of the haploid spermatid and requires acrosome development, nuclear condensation and elongation followed by elimination of the cytoplasm (Parvinen and Ruokonen, 1982). Reduction of cytoplasmic volume is accomplished mainly by the elimination of water from the cell via membrane pumps (Sprando and

Russell, 1987). Specialised structures formed between the Sertoli cells and the head region of late spermatids (tubular lobular complexes) are known to play a role in cytoplasmic elimination.

1.2.1.2. Sertoli Cells

Sertoli cells are the only somatic cellular component of the seminiferous epithelium. In the adult testis Sertoli cells are tall, columnar and stellate in appearance with their base attached to the basement membrane of the seminiferous epithelium and their apex reaching to the tubular lumen. Mature Sertoli cells provide both physical and nutritive support to developing germ cells via the numerous lateral and apical veil like processes extending between and around every germ cell (Russell, 1993b). Germ cells are dependent on lactate from the Sertoli cells for energy metabolism as they are unable to utilise glucose directly (Jutte *et al.*, 1982). The low levels of rough endoplasmic reticulum and storage granules within their cytoplasm appear contradictory to the secretory role of Sertoli cells. However Bardin (1994) (Bardin *et al.*, 1994) has proposed that this is because Sertoli cell proteins are secreted immediately following synthesis rather than being stored in vesicles.

Sertoli cells carry out a number of important functions within the seminiferous tubule. Due to the presence of tight junctions between adjacent Sertoli cells (the blood testis barrier see section 1.2.1.3) the meiotic germ cells located within the adluminal compartment are totally dependent on factors produced or transported by the Sertoli cells. The seminiferous tubule fluid (STF) produced by Sertoli cells is involved in the transport of nutrients and chemical messengers to the germ cells and spermatozoa in the adluminal compartment. STF also enables cell-cell communication to occur within the seminiferous epithelium and is responsible for the transport of spermatozoa into the epididymis (for review see (Russell, 1993a). Sertoli cells are also actively involved in the release of spermatozoa from the seminiferous epithelium. Following disengagement of the spermatid head from the specialised tubolobulbar complexes of the Sertoli cell, the surplus cytoplasm or residual body is retained in the adluminal compartment and phagocytosed by the Sertoli cell (for review see (Russell, 1993a). Sertoli cells also produce numerous proteases which may be involved in tissue remodelling during germ cell

movement in the seminiferous epithelium and growth factors such as transforming growth factor β (TGFβ) and insulin like growth factor-1 (IGF-1) which are known to be involved in germ cell proliferation and development (Parvinen, 1993). Thus it is generally accepted that germ cells rely on Sertoli cells for all their requirements for growth and differentiation. This has been further illustrated by in vitro studies that show the poor survival of spermatogenic cells when cultured in the absence of Sertoli cells (Tres and Kierszenbaum, 1983).

1.2.1.3. Blood Testis Barrier

Both physiological (Vitale *et al.*, 1973) and morphological studies (Dym and Fawcett, 1970) have documented the presence of a permeability barrier surrounding seminiferous tubules of mature mammalian testis. The most effective component of the barrier are tight junctions or desmosome like structures present between adjacent Sertoli cells (Dym and Fawcett, 1970). Penetration of electron dense markers from the blood vasculature into the seminiferous tubules is partly retarded by peritubular myoid cells (de Kretser and Kerr, 1994) and endothelial linings of blood vessels and lymphatics provide a further partial barrier (Holash *et al.*, 1993; Ploen and Setchell, 1992). The fluid composition in the seminiferous tubules is dramatically different from that in testicular lymph and blood. Free movement of proteins into the testis is restricted by the blood testis barrier and the majority of the proteins in the tubule fluid are secreted by the Sertoli cells (Mather *et al.*, 1983).

The barrier also maintains a gradient of ions, small molecules and proteins between blood and tubular fluid and sequesters the immunogenic germ cells from the body's immune system ensuring a unique environment for the developing germ cells (Setchell, 1980; Setchell *et al.*, 1994). The exclusion of small molecules from the seminiferous tubule by the blood testis barrier protects the developing germ cells from mutagenic agents (Bardin *et al.*, 1994). The production of seminiferous tubule fluid (STF) also facilitates sperm transport along the tubules towards the epididymis (Saez, 1994). The presence of inter-Sertoli cell junctions plays an important role in maintaining the STF filled tubular lumen of the seminiferous tubule.

1.2.2. Interstitium

The organisation of the interstitial space is consistent with its main endocrine function; a rich vascular supply is in close association with steroid producing Leydig cells allowing the passage of steroids into the general circulation.

1.2.2.1. Leydig Cells

There are approximately 22 million Leydig cells/adult rat testis (Mendis-Handagama *et al.*, 1987) organised into continuous strings or sheets of cells which surround the seminiferous tubules and blood vessels (Clark, 1976). They have numerous surface projections which enable them to interact with neighbouring Leydig cells and macrophages. The Leydig cell cytoplasm contains a high proportion of smooth endoplasmic reticulum which together with the numerous coated pits and vesicles found on their surface are characteristic of a highly active steroid-producing and secreting cell (Russell, 1996). Androgens are the principal steroids secreted by the Leydig cell and testosterone is the most important of these androgens (Saez, 1994).

1.2.2.2. Macrophages

Testicular macrophages are in close physical association with Leydig cells. Cytoplasmic digitations extend from Leydig cells into membrane invaginations of macrophages which suggests that these cells are functionally related (Hutson, 1994; Miller *et al.*, 1983). Degenerating Leydig cells are engulfed and phagocytosed by neighbouring macrophages (Kerr *et al.*, 1985). The latter cell type also metabolises steroids secreted by Leydig cells (Miller *et al.*, 1983). It is estimated that there is one macrophage to every four Leydig cells in the rodent testis (Hutson, 1994) and approximately 14-16% of interstitial cells are macrophages (Niemi *et al.*, 1986).

1.2.2.3. Vasculature

Blood vessels within the testis are the main route for transport of nutrients, secretory products and oxygen into the testis, as well as the removal of waste products and the transport of testicular secretory products to the rest of the body. Testicular capillaries show differences in permeability when compared to blood vessels in other organs. This difference in permeability is due to the continuous unfenestrated endothelium covering the outside of the capillaries

(Damber and Bergh, 1992a). Testicular blood flow needs to be tightly controlled as the oxygen levels within the testes are close to those that occur during hypoxia. Testosterone is thought to play an important role in the control of blood flow as a positive correlation exists between testicular blood flow and testosterone secretion (Damber and Bergh, 1992a; Setchell, 1990). Capillaries within the testis show rhythmical variations in blood flow and this is termed 'vasomotion' (Bergh and Damber, 1993). Testosterone effects on testicular capillary vasomotion may be modulated via androgen receptors expressed on blood vessel walls (Damber and Bergh, 1992b).

1.3. Hypothalamo-Pituitary-Gonadal axis

Regulation of testicular function in the adult male is under the control of the hypothalamic-pituitary-gonadal axis comprising a classical negative feedback system combined with local feedback loops involving paracrine and autocrine interactions. The endocrine interactions between the pituitary and testis are summarised in Fig 1.3. Briefly, the positive drive for the system comes from gonadotropin-releasing-hormone (GnRH) which is secreted from the terminals of the hypothalamic GnRH neurones. Binding of GnRH to GnRH receptors on gonadotrophs in the anterior pituitary stimulates the release of follicle stimulating hormone (FSH) and luteinising hormone (LH) into the circulation. FSH and LH are members of the glycoprotein hormone family and are heterodimeric, sharing a common α subunit but different hormone specific ß subunits. LH interacts with specific LH receptors present on Leydig cells and stimulates the production of testosterone (Klinefelter and Kelce, 1996). FSH interacts with specific receptors located on Sertoli cells and there is some debate as to whether spermatogonia also express functional receptors for FSH (FSHR) (Baccetti *et al.*, 1998; Rannikko *et al.*, 1996). Ligand activation of FSHR stimulates the production of inhibin B (Byrd *et al.*, 1998) by Sertoli cells. Testosterone and inhibin B diffuse into the general circulation where testosterone acts as a negative regulator for the system by inhibiting the secretion of GnRH from the hypothalamus which subsequently reduces production of LH by the gonadotrophs. In addition, increased inhibin B levels in the general circulation specifically inhibit FSH production by the anterior

pituitary. Testosterone has also been shown to negatively regulate FSH production by gonadotrophs (Pierik *et al.*, 1988).

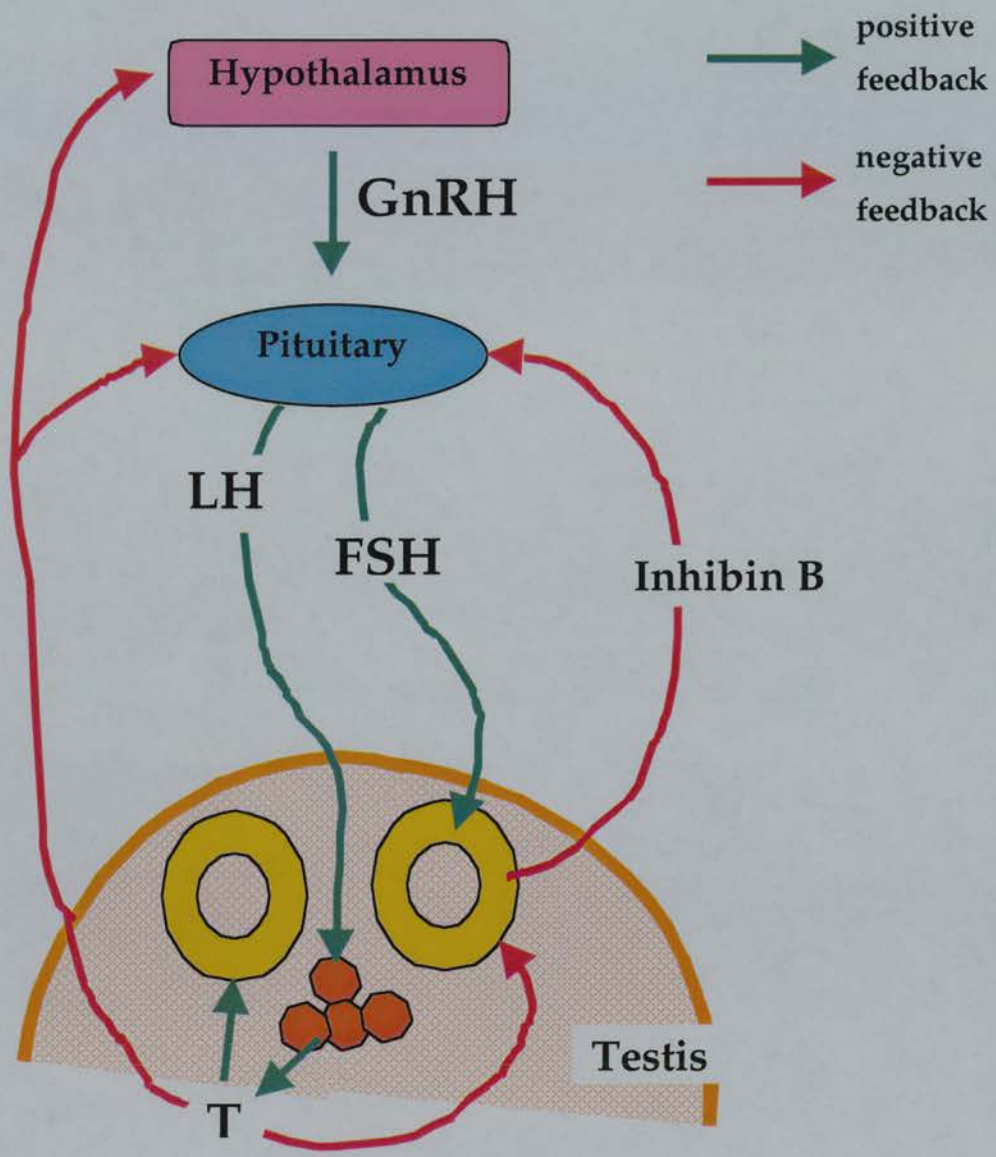

Figure 1.3: Endocrine interactions within the male reproductive axis. Seminiferous tubules are represented by yellow circles and interstitial cells are represented by orange octagons.

1.4. Fetal Development of the Testis

In mammals, the genetic sex of the embryo is determined at fertilisation, with the inheritance of either an X or Y chromosome from the father. A female phenotype is obtained following fertilisation of the ovum with a sperm

containing an X chromosome whilst a male phenotype develops following fertilisation with a Y carrying sperm. Phenotypic sex is not established until the gonads have started to develop and this occurs around midgestation in rodents (Kaufman, 1992) whilst in humans it is observed during the first trimester (Gustafson and Donahoe, 1994).

Gonads form within the developing urogenital system which is divided into the pronephros, mesonephros and the metanephros. Gonadal ridges arise from a thickening of the ventrolateral surface of each mesonephros and are first visible on postcoital day 11.5-12.0 in the mouse, day 13-13.5 in the rat and day 28-42 in the human (Bogan and Page, 1994; Gustafson and Donahoe, 1994; Kaufman, 1992). Both male and female gonads are morphologically identical and bipotential at this point of development. However a day later in the male gonad epithelial strands extend into the underlying mesenchyme forming rudimentary testicular cords, which later differentiate into the seminiferous tubules. This occurs around postcoital day 13 in the mouse (Kaufman, 1992), day 14 in the rat (Mittwoch *et al.*, 1969) and day 42 in the human (Gustafson and Donahoe, 1994). Within newly formed testicular cords, epithelial cells derived from the coelomic epithelium (Karl and Capel, 1998) begin to surround the primordial germ cells that have migrated from the wall of the yolk sac (Chiquoine, 1954; Witschi, 1948). Almost immediately the epithelial cells begin to express anti-Mullerian hormone (AMH) and are now recognised as Sertoli cells (Gustafson and Donahoe, 1994). Within the next few days mesenchymal cells between the testicular cords differentiate into steroid producing Leydig cells (Saez, 1994). Testosterone is first detectable in fetal mouse testis on postcoital day 14-14.5, in the rat testis on day 15.5 and in the human on day 50-55 (Siiteri and Wilson, 1974; Warren *et al.*, 1972; Warren *et al.*, 1975).

Two of the somatic cell lineages present in the undifferentiated gonad have a bipotential cell fate which is dependent on the sex of the organ formed: the supporting cell lineage will differentiate into Sertoli cells in the male or granulosa cells in the female, and the connective cell lineage will develop into peritubular myoid cells or stomal cells in the male and female, respectively. It is suggested that the sex determining gene (SRY), located on the Y chromosome,

triggers cell fate and differentiation of Sertoli cells which in turn directs the differentiation of other cell lineages within the gonad, along the male pathway.

1.5. Prepubertal Development of the Testis

Postnatal testicular development in mammals varies considerably between species and is dependent upon the maturity of testis at birth and on the length of time between birth and the acquisition of sexual maturity. In the rat preparation of the testis for puberty commences almost immediately after birth. In contrast, human testes change little in appearance between birth and 7-9 years of age when the spermatogenic process begins and spermatozoa are released around 11-13 years of age (Muller and Skakkebaek, 1983).

1.5.1. Germ Cells

In the prenatal rat testis gonocytes proliferate between fetal day 14 and 16 (Hilscher *et al.*, 1974), afterwhich cell division ceases and prespermatogonia are held in the G1 mitotic phase (Byskov, 1986; Vergouwen *et al.*, 1991). Mitosis of prespermatogonia resumes around postnatal day 3 and germ cells contact the basal lamina a day later (postnatal day 4). At the onset of spermatogenesis, prespermatogonia differentiate into type A and type B spermatogonia and then enter meiosis (Clermont and Perey, 1957; Vergouwen *et al.*, 1991). A high rate of selective degeneration of dividing germ cells occurs during the first wave of the spermatogenic process (Russell *et al.*, 1987) and it has been suggested that this is related to the "inexperience" of the Sertoli cell at this time. The cell types most usually observed to be undergoing degeneration within the pubertal seminiferous cords are generally those cell types that are most developmentally advanced (Russell *et al.*, 1987).

FSH plays an important role in the initiation of spermatogenesis particularly in the differentiation of type A spermatogonia into preleptotene spermatocytes. Rats treated from birth with FSH antiserum have reduced numbers of preleptotene spermatocytes and step 7-8 spermatids whilst type A spermatogonia numbers remain unchanged (Chemes *et al.*, 1979). Experiments show that both FSH and LH are required for quantitatively normal spermatogenesis (Chemes *et al.*, 1979; Dym and Raj, 1977; Raj and Dym, 1976).

The gonadotropins work synergistically (Russell *et al.*, 1987), LH by stimulating increased Leydig cell testosterone production and FSH through its direct and indirect action on both Sertoli (for review see (Sharpe, 1994) and Leydig cells (Kerr and Sharpe, 1985), respectively. It has been proposed that between days 20 and 40 the regulation in Sertoli cell function changes from one controlled by FSH to one controlled by testosterone (Sharpe, 1994). Nonetheless, these factors are thought to be required for quantitative spermatogenesis to occur in the pubertal rat (Russell *et al.*, 1987; Sharpe, 1994).

1.5.2. Sertoli Cells

1.5.2.1. Proliferation of Sertoli Cells

The number of Sertoli cells that proliferate during prepubertal development is critical to the ultimate spermatogenic potential of the adult testis due to the fact that Sertoli cells can only support a finite number of germ cells as they progress through the spermatogenic cycle (Russell and Peterson , 1984; Russell *et al.*, 1990). Using cytosine arabinoside, an antimitotic drug, Orth et al. (1988) significantly reduced the level of Sertoli cell proliferation in neonatal rats. As a result of treatment Sertoli cell numbers were reduced by 54% and they observed that the number of round spermatids was reduced by 55% in the seminiferous tubules of the treated rats after puberty. However, the number of spermatids per Sertoli cell was identical to that in controls providing powerful evidence that the production of normal numbers of germ cells in the adult rat depended, in part, on the size of the Sertoli cell population that was established during the perinatal period of life.

In the rat, Sertoli cells begin to proliferate in the testis on fetal day 16. Orth (1982) observed that maximum Sertoli cell proliferation rates were reached by fetal day 20 after which the percentage of Sertoli cells which incorporated ^{3}H-thymidine steadily decreased until day 21 and after this point dividing Sertoli cells were no longer detectable. Therefore the greatest expansion in Sertoli cell numbers occurs just prior to birth and proliferation is then on the decline. Steinberger and Steinberger (1971) and Clermont and Perey (1957) found similar results and reported that the sharpest drop in proliferation occurred after day 10 and that no labelled Sertoli cells were observed after day 16. These

results were further supported by work from Meachem, (1996) who observed that incorporation of bromodeoxyuridine (BrDU) into rat Sertoli cells sharply declined after the first 10 days of life. Cultured Sertoli cells removed from day 16 rats have been shown to incorporate tritiated thymidine but this was not observed in vivo (Steinberger and Steinberger, 1971). In the rat the number of Sertoli cells increases from 1.5 million/testis at birth to 30 million/testis at day 20 (Zhengwei *et al.*, 1990).

In the rat once Sertoli cell division ceases on, or about day 16 numbers are fixed and remain so throughout adultlife (Clermont and Perey, 1957; Steinberger and Steinberger, 1971; Wang *et al.*, 1989). Similar observations have been made in a number of mammals including the mouse where Sertoli cell proliferation stops on about day 12 with thymidine incorporation declining 100 fold between day 6 and day 12 (Kluin *et al.*, 1984), although there are some studies which state low levels of Sertoli cell proliferation take place in the rat as late as 5-7 weeks after birth (Nagy, 1972).

1.5.2.2. Regulation of Sertoli Cell Number

The regulation of postnatal Sertoli cell proliferation and maturation is primarily controlled by FSH, and thyroid hormones, respectively. Following hemicastration of rats compensatory Sertoli cell proliferation can be observed in the remaining testis. (Cunningham *et al.*, 1978; Orth, 1984; Ultee-van Gessel *et al.*, 1985). Hemicastration is accompanied by elevated FSH levels in the blood (Cunningham *et al.*, 1978) and coadministration of antibodies against FSH prevents compensatory proliferation occurring. However, if hemicastration is carried out after the main period of Sertoli cell proliferation there is no further increase in Sertoli cell number (Orth, 1984). Meachem et al., 1996 examined the effect of recombinant FSH on postnatal Sertoli cell proliferation and reported that a significant increase in Sertoli cell number occurred following neonatal FSH administration. However the duration of proliferative activity was unaffected by FSH. In vitro studies show that administration of FSH to Sertoli cells increases mitotic activity and incorporation of ^{3}H thymidine. The response to FSH is greatest in cells removed from day 10 or younger rats (Griswold *et al.*, 1977; Solari and Fritz, 1978). FSH receptors are first detectable in fetal rat Sertoli cells on fetal day 17.5 and reach maximal levels by fetal day 20-21, a time

when Sertoli cell proliferation is at its maximal rate (Warren *et al.*, 1984). FSH receptor levels in the testis remain constant during the period of Sertoli cell mitosis and the number of receptors increases with subsequent Sertoli cell maturation (Bortolussi *et al.*, 1990).

Thyroid hormones have been shown to play a critical role in 'kick-starting' the onset of Sertoli cell maturation and this in turn affects the duration of Sertoli cell proliferation. Neonatal hypothyroidism induced by the administration of 6-N-propyl-2-thiouracil (PTU) causes retardation of functional and morphological differentiation of Sertoli cell and prolongs the period of Sertoli cell replication. The net result is a significant increase in the number of Sertoli cells and consequently macroorchidism. (Hess and Cooke, 1992; Kirby *et al.*, 1992; van Haaster *et al.*, 1992). The reverse situation occurs with neonatal administration of triidothyronine (T_3) which causes hyperthyroidism. Van Haaster and coworkers (1993) treated newborn rats with T_3 for 16 days. By the end of the first week of life Sertoli cell proliferation had sharply decreased and on day 12 mitosis had stopped completely. This resulted in a 50% decrease in Sertoli numbers when compared to controls. Thyroid hormones are believed to act directly on Sertoli cells via receptors which are expressed at high levels during fetal and neonatal life. Receptor levels then decline during Sertoli cell maturation (van Haaster *et al.*, 1993). Thyroid hormones have been shown to stimulate functional maturation of Sertoli cells in culture (Palmero *et al.*, 1989; Palmero *et al.*, 1990). Therefore low T_3 levels delay Sertoli differentiation and extend the period of proliferation whilst high T_3 levels accelerate the process of maturation. Thyroid hormones have been shown to increase metabolic processes in isolated immature Sertoli cells and it has been suggested that T_3 simply increases the metabolic activity of Sertoli cells in preparation for the onset of spermatogenesis (Ulisse *et al.*, 1992).

A number of growth factors have been shown to influence Sertoli cell proliferation. Epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin and insulin like growth factor-1 (IGF-1) have mitogenic effects on immature porcine Sertoli cells (Jaillard *et al.*, 1987). Glial cell line derived neurotropic factor (GDNF) is highly expressed in the testis during postpubertal development. Coadministration of GDNF with FSH causes a dramatic increase

in the rate of replication of immature rat Sertoli cells (Hu *et al.*, 1999). FSH also works synergistically with activin to increase proliferation of Sertoli cells removed from day 9 rats. However this effect did not occur in cells recovered from day 18 rats (Boitani *et al.*, 1995). Inhibin B on the other hand is thought to have a negative autocrine effect on Sertoli cell replication via an inhibitory action on pituitary FSH secretion. FSH stimulates the secretion of inhibin B by Sertoli cells and intratesticular inhibin levels are maximal during the period when Sertoli cells are mitotically active; levels decrease thereafter (for review see (Sharpe, 1994).

The opiate peptide β-endorphin has also been shown to have a proliferative effect on Sertoli cells. Administration of opiate antagonists to immature testis had a suppressive effect on replicating Sertoli cells and their function (Kilpatrick and Rosenthal, 1986; Orth, 1986; Orth and Boehm, 1990). This is thought to be mediated by a direct action on opiate receptors expressed by Sertoli cells (Fabbri *et al.*, 1985). β-endorphin is secreted by Leydig cells (Fabbri *et al.*, 1988) and in vitro studies have shown a tropic effect of Leydig cells when cocultured with immature porcine Sertoli cells (Reventos *et al.*, 1989). It has therefore been suggested that Leydig cell secretion of β-endorphin can act as a paracrine regulator of Sertoli cell proliferation during the neonatal period.

A number of other genes have been proposed as being involved in Sertoli cell replication. For example, the fragile X mental retardation gene (FMR-1) which is silenced in patients with fragile X syndrome is associated with development of macroorchidism. In studies on mice Slegenhorst-Eegdeman et al. (1998) found that the FMR-1 gene acted as a 'brake' on the rate of Sertoli cell proliferation. Notably, Fmr-1 knock out mice had an increased rate of Sertoli cell proliferation and this was not associated with an increase in FSH or FSHR levels. Chubb (1992) suggested that there are at least two other autosomal testis–size determining genes which control testis size by regulating Sertoli cell numbers.

1.5.2.3. Maturation of Sertoli Cells

During postnatal development Sertoli cells undergo structural and functional changes some of which are related to general cellular maturation, other changes are transitional and restricted to prepubertal Sertoli cells only. As development progresses the volume of the individual Sertoli cell increases: between day 19 and day 26 rat Sertoli cell volume is doubled and this is associated with a significant increase in cell surface area. In the human testis there is an eight fold increase in cell volume during postnatal development. This increase in volume is mainly due to a large expansion in cytoplasmic volume which is accompanied by extensive development of smooth endoplasmic reticulum, golgi apparatus and mitochondria. Sertoli cells become elongated and develop finger like cytoplasmic extensions. Concurrent with the onset of spermatogenesis these elaborate cytoplasmic processes are further developed until they extend from the tubule base to the lumen thus enabling Sertoli cells to reach the increasing layers of germ cells. Cytoplasmic processes also extend to contact neighbouring Sertoli cells allowing communication to occur between adjacent cells. Once Sertoli cells have ceased dividing, the nuclear volume and nuclear surface area significantly increased as the Sertoli cells continue to mature (Gondos and Berndston, 1994). An associated rise in RNA:DNA ratio is observed in Sertoli nuclei between days 19 and 26 (Lamb *et al.*, 1982). At the same time there is an increase in cellular protein:DNA ratio and this again is indicative of a maturing Sertoli cell (Sanborn *et al.*, 1986).

1.5.2.4. Formation of the Blood-Testes Barrier

Another indicator of Sertoli cell maturation is the establishment of occluding tight junctions between Sertoli cells. These inter Sertoli cell junctions are major contributors to the blood-testes barrier and appear between adjacent Sertoli cells near to the basal lamina. In different species the timing of inter-Sertoli cell junction formation is dependent upon the onset of puberty. Thus in humans it occurs between 11 and 14 years (Nistal and Paniagua, 1983; Schukze, 1984) whilst in the rat there is gradual restricted penetration of tracers into the tubular lumen between day 16 to 19. Inter-Sertoli cell junction formation in the rat therefore occurs at the same time as Sertoli cell maturation begins (Hinton and Setchell, 1993; Russell *et al.*, 1989).

Tight junction formation begins once Sertoli cells have ceased dividing and have started the process of differentiation. If maturation is delayed as happens during hypothyroidism, occluding junction formation is also delayed and the blood testis barrier is not formed until Sertoli cells have ceased proliferating around day 30 (van Haaster *et al.*, 1992). The reverse situation occurs during hyperthyroidism where the formation of the blood testis barrier is observed as early as day 12 and this is in association with the early onset of spermatogenesis and cessation of Sertoli cell mitosis (van Haaster *et al.*, 1993).

The appearance of the tubule lumen occurs at around the same time as formation of inter-Sertoli cell junctions. Lumen formation is dependent upon seminiferous tubule fluid (STF) secretion by the Sertoli cell. STF is essential for normal spermatogenesis as it represents the main route by which nutrients and information are passed to the developing germ cells from the Sertoli cell and the basal compartment (Hinton and Setchell, 1993). Increasing testosterone levels and the appearance of elongate spermatids are believed to play a role in increasing STF production (Jegou *et al.*, 1984; Sharpe, 1989) but it has also been suggested that increased STF secretion simply reflects the increased activity of Sertoli cells during puberty (Sharpe, 1994).

Associated with Sertoli cell maturation is a down regulation in functions linked to the fetal/neonatal period of life including a decrease in AMH production and aromatisation of testosterone into oestrogen (Kuroda *et al.*, 1990; Rosselli and Skinner, 1992) coupled with an upregulation in functions predominantly associated with Sertoli cells in adulthood e.g. secretion of STF (Jegou *et al.*, 1983). These changes are concurrent with a progressive switch from mainly FSH modulation to mainly testosterone modulation. The control of this process is not fully understood. However increased numbers of germ cells have been shown to reduce the responsiveness of Sertoli cells to FSH in vitro (le Magueresse and Jegou, 1988) and FSH has been shown to increase expression of AR (Blok *et al.*, 1992; Verhoeven and Caillaeu, 1988).

1.5.3. Leydig Cells

It is generally accepted that Leydig cell differentiation in the rodent testis involves differentiation of both fetal- and adult-type Leydig cells. Fetal type

Leydig cells are responsible for masculinisation of primary sexual characteristics during fetal and neonatal life and the adult type population is responsible for pubertal masculinisation. The pig and human male have triphasic patterns of Leydig cell differentiation which comprise of fetal, pubertal and adult type Leydig cell populations (Prince, 1990; Whitehead, 1905).

In the rat, fetal type Leydig cells originate from somatic mesenchymal cells which start to differentiate and produce testosterone around fetal day 15.5. After birth a few fetal type Leydig cells may persist as a stable population through postnatal life (Mendis-Handagama *et al.*, 1987). However the relative number and cell volume of fetal type cells is reduced in the first two weeks of postnatal development.

Adult type Leydig cells differentiate from peritubular mesenchymal cells (Ariyaratne *et al.*, 2000) and Leydig cell progenitors are first seen in the postnatal testis of the rat around day 10 (Saez, 1994). Thereafter they undergo rapid proliferation which begins to slow down on postnatal day 14. At this time Hardy et al (1989) observed the start of morphological and functional differentiation of Leydig cell progenitors into adult type Leydig cells. This process occurred between postnatal days 14-28 and was linked to increased cellular production of steroidogenic enzymes and LH receptors. After day 28 Leydig cells become morphologically indistinguishable from adult type cells. Both thyroid hormone and androgens play critical roles in the differentiation, but not the proliferation of Leydig cell precursors into mature type Leydig cells (for review see(Ge *et al.*, 1996) whilst LH delays the onset of mesenchymal cell differentiation into Leydig cells (Ariyaratne *et al.*, 2000).

Oestrogen has been shown to have an inhibitory effect on both neonatal Leydig cell proliferation and development (Abney and Carswell, 1986; Abney and Myers, 1991). Both precursor and mature Leydig cells express oestrogen receptor alpha (ERα) mRNA. However between days 10 and 21 ERα mRNA levels in the progenitor Leydig cells are greatly reduced. This decline occurs at the same time as a decline in oestrogen production by Sertoli cells and it has been proposed that this decrease in oestrogen production allows precursor Leydig cells to undergo proliferation and differentiation (Zhai *et al.*,

1996). Adult type Leydig cells synthesise oestrogen which may prevent further proliferation and limit Leydig cell population growth in the adult testis (Sharpe, 1993).

1.5.4. Other Interstitial Cells and Vasculature

1.5.4.1. Macrophages

Macrophages are first detected in the fetal rat interstitium on day 19 (Hutson, 1990). After birth their rate of proliferation increases resulting in a large expansion in macrophage number and volume by the third week of life. A further rise in cell division and an increase in cell size results in a 15-20 fold increase in macrophage volume by day 47 (Hardy *et al.*, 1989; Hutson, 1990; Mendis-Handagama *et al.*, 1987). Raburn et al. (1991) have proposed that the postnatal increase in macrophage proliferation is induced by LH.

1.5.4.2. Peritubular Myoid Cells

The majority of myoid cell proliferation takes place in the fetal testes and the rate of proliferation gradually declines during the first four weeks of life. Before birth, myoid cells are in contact with the basal lamina of the seminiferous cords and the myoid cells become elongated along the basal membrane during postnatal life. As cytodifferentiation takes place, myoid cells become flatter with an expanded cytoplasm and a squamous appearance resulting in an increase in the peritubular surface (Palombi *et al.*, 1992; Vergouwen *et al.*, 1991). Palombi et al (1992), have suggested that myoid cell maturation occurs early during postnatal life with the expression of known contractile cell markers and changes in cytoarchitecture finalised by the third week of life in rats. It is thought that differentiation of myoid cells is in part due to androgen action (Skinner, 1991).

1.5.4.3. Vasculature

In the fetal testis the majority of the vasculature consists of small blood vessels. As development progresses larger blood vessels begin to develop associated with an increase in the proliferation of endothelial, perivascular and vascular muscle cells during the first two weeks of postnatal development (Mendis-Handagama *et al.*, 1987; Vergouwen *et al.*, 1991). As a result, the absolute volume of vasculature in the mature testis is 100 fold greater than at birth. At

puberty, capillaries in the rat testis develop a high level of alkaline phosphotase activity, express receptors for IGF-1 and around day 30 there is a large increase in capillary permeability to albumin and γ-globulin (Setchell *et al.*, 1994). Both connective tissue and lymphatic endothelial cells within the interstitium follow a similar pattern of development (Mendis-Handagama *et al.*, 1987; Vergouwen *et al.*, 1991).

1.5.5. Paracrine Interactions in the Prepubertal Testis

During prepubertal development numerous paracrine interactions occur between all cell types in the testis. For example, Sertoli cells mediate the effects of FSH on Leydig cell differentiation (Kerr and Sharpe, 1985) as well as functionally enhancing Leydig cell responses to LH (Chen *et al.*, 1976; Chen *et al.*, 1977). Some of the paracrine interactions between Sertoli and Leydig cells have been documented. For example, DNA synthesis in Leydig cell progenitors is stimulated by a 30kDa protein secreted by immature Sertoli cells following stimulation with FSH and testosterone (Ojeifo *et al.*, 1990). In addition, Mendis-Handagama et al. (1998) and Teerds et al. (1998) have shown that thyroid hormones trigger the differentiation of mesenchymal cells into progenitor/immature Leydig cells. Thyroid hormone receptors have been detected in Sertoli cells but not in Leydig cells during neonatal development suggesting that the observed effects are indirect and that they could be mediated via the Sertoli cells.

FSH also appears to upregulate the steroidogenic activity of Leydig cells by stimulating the production of paracrine factors by Sertoli cells. For example, FSH increases LH receptor numbers and testosterone production (Kerr and Sharpe, 1985; Vinko *et al.*, 1991) through an increase in the activity of various steroidogenic enzymes (O'Shaughnessy *et al.*, 1992). These effects may in part be mediated via insulin like growth factor (IGF-1) as FSH has been shown to increase the production of IGF by Sertoli cells and levels of IGF-1 receptors in Leydig cells increase following exposure to LH. In addition, IGF-1 increases LH receptor numbers in Leydig cells and further upregulates steroidogenic enzyme levels (Saez, 1994). Immature Leydig cells cocultured with different quantities of Sertoli cells show a 'dose dependent' upregulation in testosterone synthesis (Perrard-Sapori *et al.*, 1987). Both tissue inhibitor of

metalloproteinase-1 (TIMP-1) and CP-2 have been isolated from FSH conditioned Sertoli cell media and shown to stimulate testosterone production in cocultured Leydig cells (Boujrad *et al.*, 1995).

Conversely, Leydig cells have a trophic effect on cultured immature Sertoli cells which could be via paracrine factors (Reventos *et al.*, 1989). In addition peritubular myoid cells have been shown to secrete factors which stimulate transferrin secretion by cultured immature Sertoli cells (Norton and Skinner, 1989). Peritubular myoid cells also regulate Sertoli cell morphology by the secretion of extracellular matrix factors (Anthony and Skinner, 1989). Different germ cell types also alter Sertoli cell function in vitro. Sertoli cell secretion of plasminogen activator is upregulated in the presence of preleptotene spermatocytes and secretion levels are maximal with the onset of meiotic activity (Vihko *et al.*, 1986). Sertoli cells isolated from day 20 rats have increased androgen binding protein secretion following stimulation by various germ cell types (le Magueresse *et al.*, 1986) and FSH stimulated oestrogen secretion by immature rat Sertoli cells in culture is inhibited by cocultured germ cells (le Magueresse and Jegou, 1988).

Thus the developing postnatal testis is a very dynamic organ requiring tight regulation of maturation in which the proliferating and differentiating Sertoli cell plays a vital role in Leydig cell differentiation and 'preparation' of the testis for the onset of spermatogenesis.

1.5.6. Steroid Production in the Prepubertal Testis

1.5.6.1. Testosterone

In the testis, Leydig cells are the main and probably the only site of androgen synthesis. The first step in androgen biosynthesis is the conversion of cholesterol into pregnenolone which is carried out by the enzyme cytochrome P450 cholesterol side chain cleavage (P450scc) (see Figure 1.4). This is the rate limiting step of testosterone production and takes place on the inner membrane of mitochondria. Cholesterol can either be obtained by *de novo* synthesis from acetate or can be transported into the Leydig cell by lipoprotein and is then ferried to the outer mitochondrial membrane by the sterol carrier protein (SCP2) (Saez, 1994; Stocco and Clark, 1994). Rapid transfer of

cholesterol from the outer to the inner mitochondrial membrane is carried out by the steroidogenic acute regulatory protein (StAR) (Clark *et al.*, 1994). Pregnenolone is then metabolised by 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450 17α- hydroxylase (P450c17) in the smooth endoplasmic reticulum to form the C19 steroids. Androstenedione is converted into testosterone by 17β hydroxysteroid dehydrogenase (17β HSD). Testosterone can be transformed into the more potent androgen, DHT by 5α–reductase or converted into oestrogen by aromatisation utilising cytochrome P450 aromatase (P450arom) which is located in the microsomes of Leydig cells and neonatal Sertoli cell cytoplasm (Payne and O'Shaughnessy, 1996; Saez, 1994).

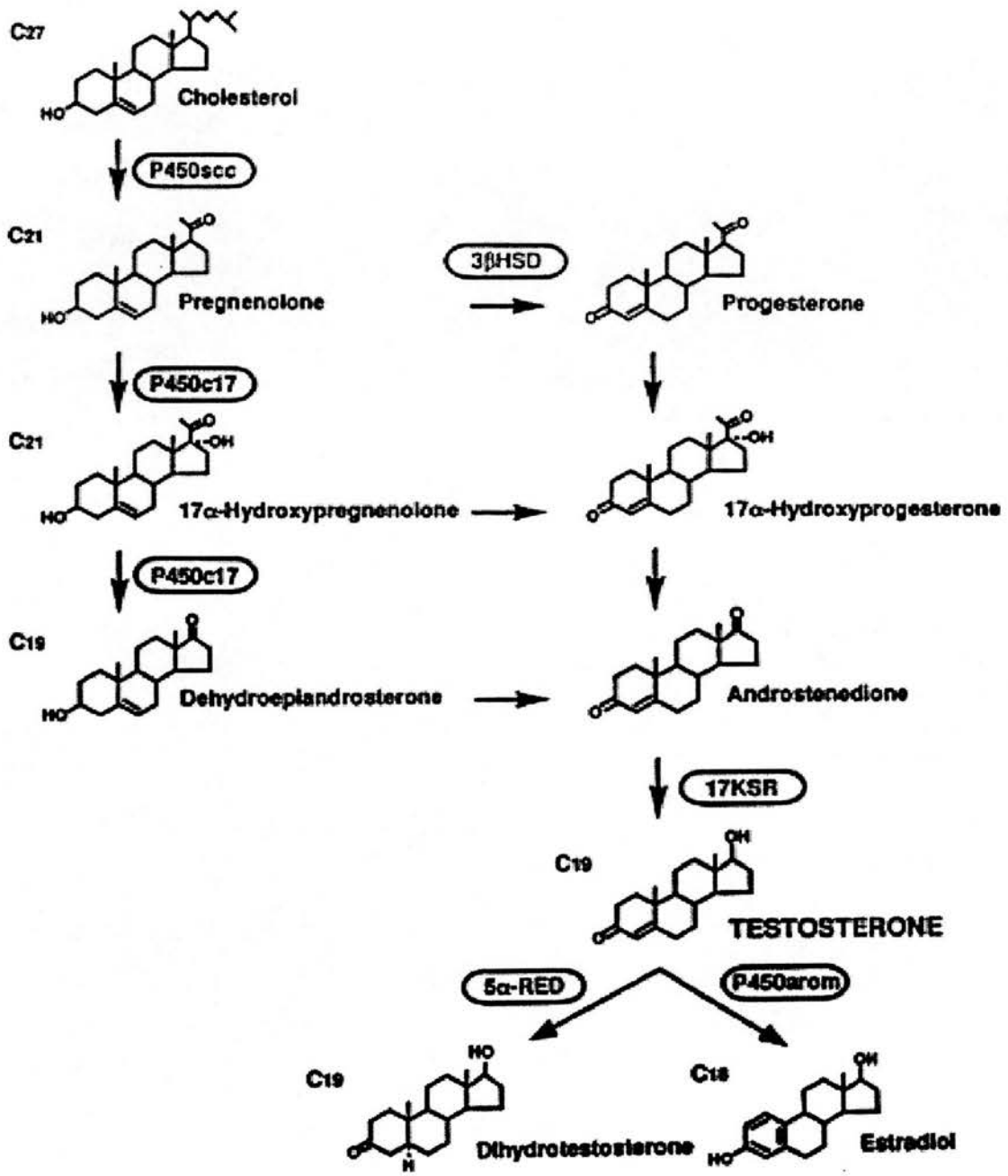

Figure 1.4: Steroid biosynthetic pathways in Leydig cells. 3ß-HSD, 3ß-hydroxysteroid dehydrogenase; 17KSR, 17ß-HSD; 5α-RED, 5α reductase. (Adapted from (Payne and O'Shaughnessy, 1996).

During postnatal development the predominant androgens secreted by the Leydig cells are 5α-androstene-3α, 17β –diol and androsterone with levels 5-8 times greater than testosterone. This is due to increased production of 5α-

reductase by differentiating Leydig cells between days 15 and 32. Testosterone becomes the predominant androgen secreted by the testis after day 45 (Dorrington and Khan, 1993; Moger, 1977). Isolated seminiferous tubules from rats at all stages of development contain 5α-reductase and have the ability to convert testosterone into DHT which can be metabolised to 5α-androstanediols and further to androsterone. 5α-Reductase and hydroxysteroid dehydrogenase activity have been shown to be significantly higher in tubules from immature rats than adult rats (Dorrington and Fritz, 1975).

The synthesis of androgens by Leydig cells is regulated by LH. Receptors for LH are coupled to adenylate cyclase and are located on the plasma membrane of Leydig cells. An acute response to LH results in an increase in the amount of cholesterol transported to the inner mitochondrial membrane purportedly via an increase in levels of StAR protein (Luo *et al.*, 1998). LH also upregulates the activity and levels of P450c17 (Klinefelter and Kelce, 1996). Decreased LH levels result in atrophy of Leydig cells, a decrease in LH receptor numbers and a loss of the ability to secrete testosterone (Saez, 1994).

A short-loop negative feedback mechanism is known to exist within the testis such that testosterone can inhibit its own biosynthesis. Darney et al (1996) (Darney *et al.*, 1996) demonstrated specific, competitive binding of testosterone to the active site of P450c17. Negative regulation of P450c17 by testosterone is also mediated through the androgen receptor (Hales *et al.*, 1987). Oestrogens are known to suppress gonadotropin release from the pituitary which results in a subsequent decrease in testosterone biosynthesis by the Leydig cells (Kalla *et al.*, 1980). Oestrogen also has a direct inhibitory affect on Leydig cell steroidogenesis via the reduction in LH receptor levels (Saez *et al.*, 1978) and competitive inhibition of P450c17 and 17βHSD activity (Brinkman *et al.*, 1980; Kalla *et al.*, 1980; Samuels *et al.*, 1964). The oestrogen concentrations used in these studies were nonphysiological: therefore further studies are required to determine if physiological levels of oestrogen have a direct effect on Leydig cell steroidogenesis.

In Sertoli cells, androgens induce a rapid but transient (15 minutes) increase in RNA polymerase II activity followed by a further and more prolonged increase

3 to 6 hours later. Isolated Sertoli cells that are deprived of testosterone undergo morphological changes in nuclear and mitochondria as well as an increase in lipid droplets and decreases in smooth endoplasmic reticulum (Dym and Raj, 1977). In vivo, the effects of testosterone are dependent upon the stage of Sertoli cell differentiation (Bardin *et al.*, 1994) and Sertoli cells have been shown to become more responsive to testosterone with increasing cell maturation. For example, production of STF is under the control of testosterone in the adult rat whereas in the prepubertal testis it is under the control of FSH. A similar change in responsiveness is true for the secretion of androgen binding protein and the control of other Sertoli cell functions (Sharpe, 1994).

1.5.6.2. Oestrogen

The microsomal enzyme cytochrome P450 aromatase (P450arom) catalyses the conversion of androgens to oestrogens (see Figure 1.5). The substrate for aromatase can be either testosterone or androstenedione. Aromatase activity can be detected in the smooth endoplasmic reticulum of cells in the liver, kidney, brain, fat and skeletal tissue (Carreau *et al.*, 1999). In the testis, expression has been observed in the Leydig cells, Sertoli cells and germ cells. In the neonatal testes of rats and mice Sertoli cells are the main site of testosterone aromatisation. However during puberty this role switches to Leydig cells where the majority of aromatase activity remains thereafter (Papadopoulos *et al.*, 1986; Tsai-Morris *et al.*, 1985).

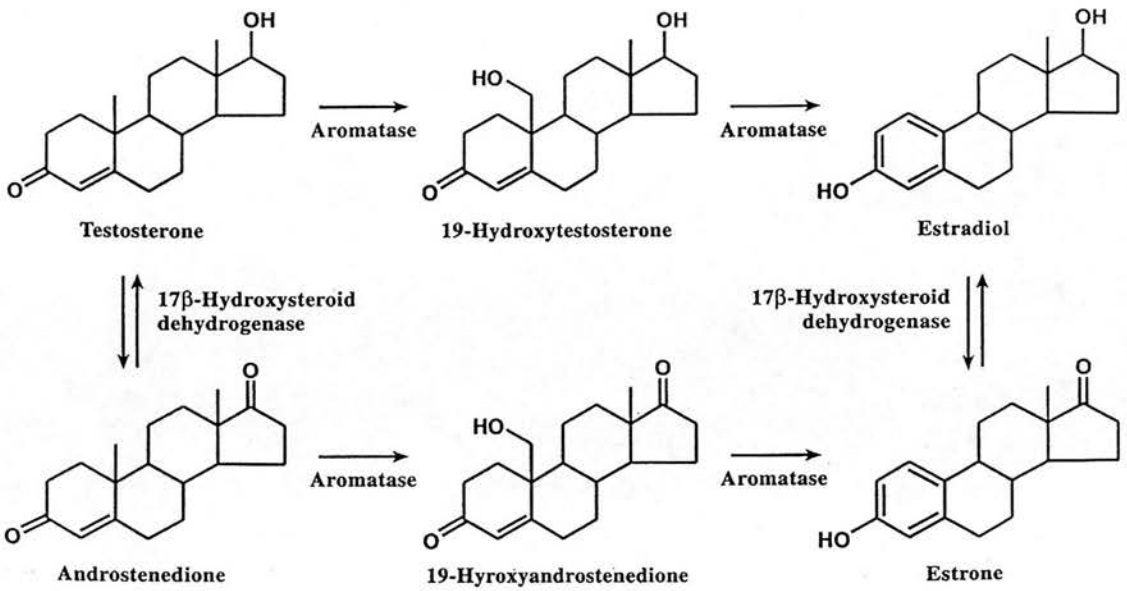

Figure 1.5: The aromatase pathway. Diagram showing conversion pathway for aromatisation of testosterone into oestradiol. (Adapted from (Sundaram and Kumar, 1996).

The synthesis of oestrogens in the immature Sertoli cell is primarily regulated by FSH. In vitro studies show that in isolated rat Sertoli cells, FSH stimulated aromatase activity decreases with increasing age. FSH induced aromatase activity is highest in Sertoli cells from rats aged day. However by day 30 only low levels of oestrogen are synthesised by the Sertoli cells even in the presence of FSH (Dorrington and Armstrong, 1979). In adult rats the expression of aromatase in Leydig cells is induced by LH. Leydig cells isolated from day 15 rats exhibit constitutive aromatase activity which becomes LH inducible by day 25 (Sundaram and Kumar, 1996).

Androgens can influence aromatase activity by serving as a substrate for oestrogen synthesis, by inhibiting basal and FSH induced aromatase activity in Sertoli cells (Verhoeven and Cailleau, 1988a), and by increasing P-Mod-S activity and secretion of a 50-100 kDa protein from peritubular cells which have both been shown to inhibit aromatase activity in Sertoli cells (Rosselli and Skinner, 1992; Verhoeven and Cailleau, 1988b). Conditioned media from spermatocytes, spermatids and stage VII-VIII seminiferous tubules also inhibits Sertoli cell aromatase activity (Magueresse and Jegou, 1988).

The physiological significance of oestrogen production in the immature testis has remained obscure. A marked drop in plasma and testicular testosterone concentrations is reported following 24 hour oestradiol benzoate treatment. However, LH levels remain unchanged. Thus it has been proposed that direct inhibition of testosterone production in Leydig cells by oestradiol is taking place. It is possible that the release of oestradiol from Sertoli cells prior to the onset of puberty serves to suppress the synthesis of androgens by Leydig cells thus holding back the onset of spermatogenesis until Sertoli cells are undergoing maturation (Dorrington and Khan, 1993).

A direct correlation between oestrogen concentrations and Sertoli cell mitotic activity has been demonstrated and it is notable that oestrogen stimulates DNA synthesis in cultured granulosa cells from immature rats. Oestradiol is thought to stimulate the production of TGF-β which interacts with FSH to promote the progression of the granulosa cells through the G1 phase of the cell cycle. FSH and TGF-β act synergistically in isolated Sertoli cells from day 10 rats to increase DNA synthesis and oestrogen is known to increase Sertoli cell secretion of TGF-β in immature pigs. It has been proposed that in the immature animal FSH acts to stimulate the synthesis of oestradiol via increased aromatase levels in Sertoli cells. Increased levels of oestradiol in turn stimulate secretion of TGF-β which together with FSH upregulates the mitotic activity of Sertoli cells. The decline in Sertoli cell responsiveness to FSH parallels a decrease in aromatase activity and oestrogen secretion is associated with a decline in TGF-β production and a consequent decline in mitotic activity of Sertoli cells (Dorrington and Khan, 1993).

Female mice deficient in aromatase (ArKO) due to targeted disruption of the aromatase gene are infertile. However male ArKO mice are fully fertile and have markedly elevated testosterone levels; the number of Sertoli cells has not been determined (Fisher *et al.*, 1998a).

1.5.7. Steroid Receptors in the Prepubertal Sertoli Cell

Modulation of cell function by steroids is mediated via specific intracellular receptors which are expressed in steroid target tissues (Carson-Jurnica *et al.*, 1990; Evans, 1988). All members of the steroid receptor superfamily share a

common arrangement of five structure-function domains (A-F) (reviewed by (Evans, 1988). The highly conserved DNA binding domain (C) is involved in DNA binding and receptor dimerisation, a less well conserved ligand binding domain (E) contains regions involved in ligand binding, receptor dimerisation, nuclear localisation and also interacts with transcription cofactors, the A/B and F domains at the N and C termini respectively, are involved in transactivation and the D region acts as a hinge contributing to the flexibility of the receptor and influencing DNA binding. Following ligand binding, steroid receptors form dimers. The steroid-ligand dimer regulates gene transcription through binding to specific sequences in the regulatory regions of target genes. These specific sequences are called response elements (reviewed in (Carson-Jurnica *et al.*, 1990; Evans, 1988).

1.5.7.1. Androgen Receptor (AR)

Androgen action is carried out in target tissues via binding to a specific AR encoded by a single gene on the X-chromosome (Chang *et al.*, 1988; Lubahn *et al.*, 1988). Immunoexpression of AR in the testis is restricted to nuclei of Sertoli cells and interstitial cells which include peritubular myoid cells, Leydig cells and vascular endothelial cells (Bremner *et al.*, 1994). It is generally agreed that germ cells do not immunoexpress AR protein. AR is first expressed in the fetal rat testis and is immunolocalised to interstitial cells however significant nuclear staining is not seen in Leydig or Sertoli cells during fetal life (Majdic *et al.*, 1995). In immature rats, peritubular myoid cell immunostaining is abundant in day 5 testis, AR immunoexpression is also detected in Sertoli cells at this age and the intensity of staining increases with increasing age. AR immunoexpression in Sertoli cells becomes stage dependent between days 21 and 35. Thus stage specificity is acquired during normal development as spermatogenesis matures (Bremner *et al.*, 1994).

The distribution of AR in the seminiferous tubule as determined by immunocytochemistry is in agreement with ligand binding measurements of AR and with in vivo studies where AR has been detected in isolated Sertoli cells, peritubular myoid cells and Leydig cells. Levels of AR are higher in Sertoli cells cultured from day 35 rat testis than Sertoli cells obtained from day 15 rats (Sar *et al.*, 1993).

Concentrations of AR in cultured Sertoli cells from day 19 rat testis increase 3-4 fold following FSH and testosterone administration. The effects of testosterone and FSH are additive. FSH also increases the abundance of AR mRNA and increases AR protein levels in the immature testis (for review see (Sar *et al.*, 1993). Androgen stimulates AR levels in immature Leydig cells but is without effect in adult Leydig cells. Conversely, AR levels in adult Sertoli cells are upregulated in response to androgen stimulation but levels remain unchanged in immature Sertoli cells (Shan *et al.*, 1997). Following rat neonatal oestrogenisation, Sertoli cell expression levels of both AR mRNA and protein are reduced (Sharpe *et al.*, 1998; Tena-Sempere *et al.*, 2000). These changes are not solely caused by decreases in gonadotropin levels suggesting that oestrogen has a direct inhibitory effect on Sertoli cell AR expression in the prepubertal rat testis. Alteration of normal germ cell complement in the mature rat testis does not modify levels of AR protein detected by immunohistochemistry (Bremner *et al.*, 1994).

1.5.7.2. Oestrogen receptor beta (ERβ)

There are two subtypes of oestrogen receptor (ER), alpha (ERα) and beta (ERβ). The classical ERα first discovered in 1958 and cloned in 1986 (Green *et al.*, 1986) was for many years believed to be the only ER to existence. However in 1996 a second ER (ERβ, (Kuiper *et al.*, 1996)) was cloned from rat prostate. In mammals, both receptors have the greatest homology in the DNA binding domain (97% amino acid identity) and the ligand binding domain (59% amino acid identity) (Enmark *et al.*, 1997; Kuiper *et al.*, 1996). Overall the degree of homology between the two receptors is quite low apart from within the DNA binding domain and they are encoded on separate chromosomes (Enmark *et al.*, 1997). In the rat additional mRNA splice variants of ERβ have been found which contain an insertion in the ligand binding domain (ERβ2) (Chu and Fuller, 1997; Peterson *et al.*, 1998).

In vitro studies have shown both receptor subtypes have similar affinities for a wide range of ligands including some synthetic oestrogens. However Scatchard analysis indicates that ERβ has a lower affinity for oestradiol than ERα. In contrast ERβ binds the 'environmental oestrogens' methoxychlor and bisphenol A in addition to the phytoestrogens genistein and coumestrol with

considerably higher affinity than ERα (Kuiper *et al.*, 1998). If both subtypes of ER are present in a single cell type following interaction with the ligand it is possible that receptors form homodimers (ERα-ERα or ERβ-ERβ) or heterodimers (ERα–ERβ) (Cowley *et al.*, 1997). The two homodimers, when complexed with oestradiol or certain anti-oestrogens, may signal in opposite ways following interaction with Fos and Jun proteins which modulate gene activation via an AP-1 site. Studies in vitro have shown that ERα, and 17β oestradiol activate transcription of a reporter construct whereas ERβ and 17β-oestradiol inhibit transcription. Moreover the antioestrogens tamoxifen, raloxifene and ICI 164384 are potent transcriptional activators with ERβ at AP1 sites (Paech *et al.*, 1997).

ERβ mRNA has been detected by reverse transcription PCR analysis in a range of rat tissues including the testis, pituitary, ovary, uterus, bladder, brain, lung, thymus and heart (Kuiper *et al.*, 1997). ERß mRNA has also been detected in adult male rat efferent ducts, prostate, vas deferens and all regions of the epididymis. Immunohistochemical studies have shown that ERß protein is widely expressed in a number of tissues, although highest levels appear to be in granulosa cells of the ovary (Saunders *et al.*, 1997). In the adult rat testis ERß is immunoexpressed in Sertoli cells, Leydig cells and some germ cells types (spermatocytes and spermatogonia) (Saunders *et al.*, 1998; van Pelt *et al.*, 1999). Pelletier et al., (2000) found ERß expression restricted to Sertoli cell nuclei in adult rat testis. In addition ERα expression was localised to the nuclei of mature Leydig cells, round spermatocytes and spermatids. This is in disagreement with other studies where testicular ERα protein expression is restricted to interstitial cells in the rat (Fisher *et al.*, 1997).

When this study was started ERβ had only recently been cloned and therefore very little information regarding expression and regulation of ERβ during development was known. Studies within the Unit demonstrated that ERß was expressed in fetal gonocytes (Saunders *et al.*, 1998) and this was confirmed by van Pelt et al., (1999) who detected ERß mRNA and protein in gonocytes and Sertoli cells in day 4 rat testis. In neonatal and immature rat testes ERß protein has been detected in Sertoli cells, Leydig cells, peritubular cells and

spermatogonia (Saunders *et al.*, 1998; van Pelt *et al.*, 1999). This pattern of ERß expression indicates that oestrogens can directly affect germ cells during testicular development and the first wave of spermatogenesis. In mice ERβ mRNA levels in testis have been reported to peak by day 12 and then decrease to undetectable levels by day 26 and ERβ protein has only been immunolocalised to neonatal spermatocytes (Jefferson *et al.*, 2000).

Neonatal exposure to oestrogen differentially alters testicular expression of ERα and ERβ mRNA levels in the developing rat. ERα mRNA levels are significantly reduced following oestradiol benzoate administration whereas expression levels of ERβ mRNA directly increase during postnatal/prepubertal development following neonatal oestrogen benzoate exposure (Tena-Sempere *et al.*, 2000). These changes in oestrogen receptor expression levels are not solely attributable to oestrogen-induced decreases in gonadotropin secretion during the critical period of neonatal differentiation (Tena-Sempere *et al.*, 2000). This work strongly suggests that neonatal oestrogen administration causes an increase in ERβ mRNA levels by acting directly on the developing testis.

Transgenic male mice with targeted disruption of the ERα gene (ERKO) are infertile indicating that ERα plays an essential role in the regulation of male reproduction. Adult ERKO mice have significantly fewer epididymal sperm than heterozygous or wild-type males. Spermatogenesis is present in some seminiferous tubules of 3-5 month old ERKO males. However other tubules either have a dilated lumen and a disorganised seminiferous epithelium with few spermatogenic cells or tubules lack a lumen and contain mainly Sertoli cells. There are no obvious differences between the seminiferous tubules of day 10 wild-type and ERKO mice but the lumen in ERKO males is dilated in all seminiferous tubules by day 20. Serum testosterone levels are elevated whereas gonadotropin levels are unaffected in the ERKO mouse when compared to wild type. Sperm from adult ERKO mice have reduced motility and are unable to fertilise an egg in vitro. In addition mutation of the ERα gene alters normal male sexual behaviour leading to reduced mating frequency. It has been proposed that lower sperm concentrations in ERKO mice are caused by dilation of the seminiferous tubules due to a reduction in fluid resorption in

the efferent ducts and epididymis (Hess *et al.*, 1997) and such dilation inhibits and disrupts spermatogenesis (Eddy *et al.*, 1996).

Furthermore it has been proposed that ERα does not regulate genes expressed in germ cells but affects sperm function indirectly through somatic cells which support spermatogenesis and/or epididymal maturation. In the mouse transplantation studies have demonstrated that germ cells do not need to express ERα for normal development or for functioning during fertilisation (Mahato *et al.*, 2000).

Transgenic mice that lack ERβ expression have also been generated (BERKO) (Krege *et al.*, 1998). These transgenic mice develop normally and are indistinguishable grossly and histologically as young adults from wild type mice. Female BERKO mice have reduced fertility whilst there are no overt abnormalities in the male BERKO mice and reproduction is normal. Thus ERβ is proposed to be essential for normal ovulation efficiency but is not essential for female or male sexual differentiation or fertility. ERα but not aromatase or ERβ is essential for normal male fertility and sexual behaviour (Krege *et al.*, 1998). It has been proposed that ERβ plays a role in the fine tuning of oestrogen actions on testicular function (Carreau *et al.*, 1999).

1.5.8. Gene expression during Prepubertal Development

1.5.8.1. Follicle Stimulating Hormone (FSH)

FSH is produced by the anterior pituitary. mRNA transcripts for both FSH subunits have also been located in spermatocytes, spermatogonia and round spermatids suggesting possible paracrine roles for FSH in the testis (Baccetti *et al.*, 1998; Markkula *et al.*, 1995). In vivo and in vitro experiments have established an essential role for FSH in Sertoli cell mitosis which takes place during fetal life and the first two weeks of postnatal life. In prepubertal rat testis FSH is also essential for final Sertoli cell maturation which includes formation of the blood testis barrier and the initiation of the first wave of spermatogenesis (Posalaky *et al.*, 1981; Solari and Fritz, 1978). FSH also differentially stimulates Sertoli cell synthesis of inhibin and SGP-1 (Sanborn *et al.*, 1986).

1.5.8.2. Follicle Stimulating Hormone Receptor (FSHR)

The biological action of FSH is initiated when FSH interacts with the FSH receptor (FSHR). It is generally accepted that FSHR expression in the male is restricted to Sertoli cells (Griswold, 1993). However there is limited evidence indicating FSHR are expressed by spermatogonia (Orth and Christensen, 1978). Full length FSHR mRNA is first detected in rat testis on fetal day 16.5 and specific FSH binding can be observed in fetal rat testis from day 17.5 onwards (Rannikki *et al.*, 1995; Warren *et al.*, 1984). FSHR mRNA can be detected in the postnatal testis and isolated Sertoli cells from rats aged between day 10 and adulthood (Heckert and Griswold, 1991). As with other polypeptide hormone receptors the FSHR has an extracellular N-terminal portion, seven transmembrane helices and an intracellular C terminal region which interacts with a Gs protein. Binding of FSH to FSHR activates the Gs protein which in turn activates adenylate cyclase, cAMP levels increase with a subsequent increase in protein kinase activity (for review see (Griswold, 1993). The overall response of Sertoli cells to FSH is complex. Increased levels of protein kinase A (PKA) result in an increase in the phosphorylation of Sertoli cell proteins. In addition increased PKA levels upregulate phosphorylation of transcription factors e.g. CREB, which in turn regulate transcription of specific Sertoli cell genes which contain cAMP response elements in their promoter regions (Griswold, 1993). Almost simultaneous with increased cAMP levels there is an observed increase in mRNA levels of the proto-oncogene *cfos.* Upregulation of *cfos* takes place within 15 minutes of FSH administration in cultured immature Sertoli cells. Thus FSH has immediate effects on Sertoli cell gene transcription as well as effects which are measurable within hours, such as the synthesis of specific Sertoli cell proteins e.g. inhibin, SGP-1 (for review see (Griswold, 1993) and Sertoli cell specific mRNA transcripts e.g. LRPR1 (Slegtenhorst-Eegdeman *et al.*, 1995). Resulting from the plethora of events which take place following FSH binding to FSHR it is difficult to determine which factors are regulated directly by FSH and which are regulated by secondary events.

Alongside Sertoli cell maturation, changes in the primary functions of FSH in Sertoli cells also arise. Phosphodiesterase (PDE) degrades cAMP into AMP. Both in vivo and in vitro research has shown an increase in PDE activity takes place following FSH administration to immature rats and Sertoli cells isolated

from prepubertal rats (Griswold, 1993). Levels of PDE in Sertoli cells have been shown to increase with increasing age such that Sertoli cells become less responsive or refractory to FSH stimulation. A marked fall in FSH-stimulated cyclic AMP production even in the presence of a phosphodiesterase inhibitor is observed with advancing age (Steinberger *et al.*, 1978). The decline in Sertoli cell responsiveness to FSH correlates with the age related diminishing effects of FSH in the testis such that by day 25 the responsiveness of the testis to FSH has already undergone a significant decline (Means *et al.*, 1980).

FSH responsiveness in cultured Sertoli cells also decreases when Sertoli cells are cocultured with pachytene spermatocytes and round spermatids (Griswold, 1993). As the responsiveness of the Sertoli cell to FSH declines during early puberty, responsiveness of Sertoli cells to testosterone increases. This is partly mediated by an increase in androgen receptors which is itself stimulated by FSH. In the adult rat testosterone is the major regulator of FSH secretion having taken over the prepubertal regulation of FSH secretion from inhibin B (Sharpe, 1994).

1.5.8.3. SGP-1

SGP-1 is a heavily glycosylated and sulphated 70 kDa protein secreted in high amounts by the seminiferous epithelium (Sylvester *et al.*, 1984). SGP-1 is the rat homologue of human prosaposin which is proteolysed into the four smaller (15 kDa) activator proteins: saposin A, B, C and D (O'Brien and Kishimoto, 1991). Saposins are found in lysosomes and solubilise certain membrane glycolipids, form complexes with lysosomal enzymes, and promote hydrolysis of glycolipid substrates (Kretz *et al.*, 1990). Sertoli cells synthesise two mature forms of SGP-1 (Igoura and Morales, 1995): an intracellular form (65 kDa) located to lysosomes, which is also found in the liver and spleen (O'Brien and Kishimoto, 1991) and a secreted form which is found in several extracellular fluids (Igoura and Morales, 1995). It is the lysosomal form of rat SGP-1 that is broken down into the smaller 15 kDa polypeptides which correspond to saposins (Igoura and Morales, 1995). The lysosomal form of SGP-1 is targeted directly to the lysosomes from the Golgi apparatus.

The lysosomes fuse with phagosomes containing cytoplasmic residual bodies detached from spermatids during spermiation. It is proposed that saposins play a role in the hydrolysis of membrane glycolipids present in phagocytosed residual bodies (Igoura and Morales, 1995; Morales *et al.*, 1998). The function of the secreted form of SGP-1 is unclear. However the 70 kDa form does interact with the plasma membrane of developing spermatids and is thought to act in glycolipid transfer between Sertoli cells and developing spermatids (Morales *et al.*, 1995). In addition the presence of SGP-1 in a variety of biological fluids suggests that secreted SGP-1 is also involved in the transport of lipids within biological fluids (Morales *et al.*, 1996).

Studies using quantitative PCR have examined changes in steady state levels of SGP-1 mRNA in whole rat testes between the ages of postnatal day 3 and day 60. SGP-1 mRNA levels increase rapidly after birth reaching maximal levels between days 10 and 20. A rapid decline in mRNA levels is observed thereafter with adult levels of mRNA similar to those in day 3 testes (Mathur *et al.*, 1994). A significantly lower amount of SGP-1 is secreted from immature seminiferous tubules when compared to amounts secreted by adult seminiferous tubules (McKinnell and Sharpe, 1997). Both in vivo and in vitro experiments have shown that SGP-1 production by adult Sertoli cells is not regulated by germ cells (Grima *et al.*, 1992; Mathur *et al.*, 1994; Sharpe *et al.*, 1993; Stallard and Griswold, 1990), although Sharpe et al., (1993)
observed a significant decrease in SGP-1 secretion by isolated seminiferous tubules following co-depletion of pachytene spermatocytes and round spermatids.

1.5.8.4. Inhibin

Inhibin is a member of the TGFβ family of growth factors. The dimeric glycoprotein is mainly produced by Sertoli cells in the adult testis and is able to modulate the expression of FSH in the anterior pituitary. Inhibin is a heterodimer composed of an α-subunit covalently linked by di-sulphide bridges to either a βA subunit (inhibin A) or a βB subunit (inhibin B). During fetal life inhibin α and βB protein subunits are expressed in fetal type Leydig cells (Majdic *et al.*, 1997). In the postnatal testis, following the replacement of fetal type Leydig cells with adult counterparts expression of both inhibin

subunits is restricted to Sertoli cells (Majdic *et al.*, 1997). Expression of inhibin βA subunit has not been detected in rat testis (Majdic *et al.*, 1997) and it has been proposed that inhibin B is the major inhibin form synthesised in the male in the human (Illingworth *et al.*, 1996), rhesus monkey (Plant *et al.*, 1997) and rat (Woodruff *et al.*, 1996). Sertoli cells are thought to be the predominant or sole source of inhibin B in circulation.

Inhibin mRNA levels are maximal between days 15 and 25 and gradually decline thereafter (Keinan *et al.*, 1989). Serum levels and immunoexpression of inhibin B are increased between day 3 and days 10-15 afterwhich levels plateau and decline with late puberty (Majdic *et al.*, 1997; Rivier *et al.*, 1988; Sharpe *et al.*, 1999). In the postnatal rat testis stage specific immunoexpression of inhibin-α and βB is observed as early as day 3 becoming fully stage dependent by day 27 (Majdic *et al.*, 1997).

In the adult rat testis stage specific expression of inhibin by Sertoli cells (Majdic *et al.*, 1997) suggests that secretion may be regulated by germ cells. Sertoli cells cultured in the presence of germ cells secrete lower levels of inhibin than isolated Sertoli cells cultured alone (Ultee-van Gessel *et al.*, 1986). Addition of early spermatids to day 20 rat Sertoli cells in culture increased inhibin production (Pineau *et al.*, 1990) and the absence of late spermatids in intact adult rat testis causes a decline in inhibin levels (Allenby *et al.*, 1991; Maddocks *et al.*, 1992).

It is well established that FSH upregulates inhibin production by Sertoli cells during early postnatal life. Between days 8 and 22 FSH and inhibin levels in rat plasma are inversely related (Rivier *et al.*, 1988). Numerous in vitro studies have demonstrated an upregulation of inhibin B protein secretion and inhibin-α mRNA levels following FSH administration or addition of factors known to raise intracellular cAMP levels (dibutryl-cyclic AMP, cholera toxin, forskolin) (Bicsak *et al.*, 1987; Keinan *et al.*, 1989; Klaij *et al.*, 1990; Toebosch *et al.*, 1989). Recently an ELISA specific for inhibin B has demonstrated that in both male humans and rats a dose and time dependent increase in inhibin B production by Sertoli cells takes place following exogenous FSH administration both in vitro and in vivo (Anawalt *et al.*, 1996; Depuydt *et al.*, 1999; Sharpe *et al.*, 1999). FSH

from the anterior pituitary regulates Sertoli cell expression of inhibin B by a classical feedback loop. Inhibin B in turn regulates FSH production by the anterior pituitary. Following castration, plasma FSH levels are increased in the immature male (Hermans *et al.*, 1980). Sharpe et al., (1999) established an association between FSH levels, Sertoli cell numbers and inhibin B plasma levels. They showed that modification of neonatal FSH levels causes a corresponding change in Sertoli cell number and testis weight in addition to a proportional change in inhibin B plasma levels.

Neutralisation of endogenous inhibin with antiserum increases plasma FSH levels in day 10 rats but has no effect in rats older than 26 days which suggests that inhibin is only important in regulating FSH production in the immature animal (Rivier *et al.*, 1988). However stage specific expression of inhibin and labelled binding of inhibin to germ cells suggests additional paracrine functions of inhibin in the adult testis (Woodruff *et al.*, 1992).

Testosterone has been shown to have either no effect or an inhibitory effect on inhibin production by Sertoli cells in culture (Bicsak *et al.*, 1987; Depuydt *et al.*, 1999; Morris *et al.*, 1988; Toebosch *et al.*, 1989) and in normal men following exogenous testosterone administration (Anawalt *et al.*, 1996). In contrast oestrogen has a stimulatory effect or no effect on inhibin production by Sertoli cells in culture (Bicsak *et al.*, 1987; Depuydt *et al.*, 1999; Morris *et al.*, 1988). Inhibin expression is also regulated by epidermal growth factor, transforming growth factor-β1, tumour necrosis factor α and insulin-like growth factor demonstrating paracrine regulation of inhibin production within the testis. (le Magueresse-Battistoni *et al.*, 1995; Morris *et al.*, 1988; Toebosch *et al.*, 1988).

1.5.8.5. GATA 1 and 4

The GATA binding proteins are a family of $CX_2CX_{17}CX_2C$ zinc finger transcription factors (Evans and and Felsenfeld, 1989; Tsai *et al.*, 1989) that bind to the *cis* regulatory sequence WGATAR (Yamamoto *et al.*, 1990) as well as related sequences CGATGG and AGATTA (Ko and Engel, 1993; Merika and Orkin, 1993). These sequences are found in promoter, enhancer and locus control regions of a number of genes involved in neonatal testis development including StAR (Silverman *et al.*, 1999), AMH (Viger *et al.*, 1998) and inhibin-α

(Feng *et al.*, 1998). There are so far six members of the vertebrate GATA family: expression of GATA-1, 4 and 6 has been observed in mammalian gonads (Ito *et al.*, 1993; Ketola *et al.*, 1999; Viger *et al.*, 1998) and Onodera et al. (1997) have also shown testicular expression of GATA2.

1.5.8.5.1. GATA-1

The predominant GATA-1 mRNA transcript found in the testis is transcribed from a testis specific distal promoter and first exon found 8kb upstream from the normal proximal promoter and first exon (Ito *et al.*, 1993). Low levels of GATA-1 mRNA and protein are observed in rat testes on postnatal day 7 and maximum levels are reached between days 14 and 23 (Viger *et al.*, 1998). GATA-1 immunolocalisation is restricted to Sertoli cell nuclei and is not observed in interstitial or germ cells (Ketola *et al.*, 1999; Yomogida *et al.*, 1994). Yomogida et al., (1994) found a similar pattern of GATA-1 expression in the mouse testis. Initially, Sertoli cell expression of GATA-1 is constitutive however by day 35 expression appears to be becoming stage dependent such that in adult testis GATA-1 protein is only observed in Sertoli nuclei during spermatogenic stages VII-IX (Yomogida *et al.*, 1994). This could explain the very low levels of GATA-1 mRNA detected in the adult mouse testis (Ito *et al.*, 1993; Onodera *et al.*, 1997) and the absence of GATA-1 protein in adult mouse testis observed by Viger et al., (1998). Maximum expression levels of GATA-1 in both mouse and rat testis coincide with the cessation of Sertoli cell proliferation and subsequent Sertoli cell maturation. Stage dependent GATA-1 expression is first observed during the onset of spermatogenesis.

Germ cells have been shown to negatively regulate GATA-1 expression in the adult mouse testis (Yomogida *et al.*, 1994). In the seminiferous tubules of c kit mutant W/W^v adult mice (which do not contain germ cells) no stage specific GATA-1 expression is observed and virtually all Sertoli cell nuclei express GATA-1 protein. A similar pattern of expression is observed in tubules of adult jsd/jsd (juvenile spermatogonial depletion) which contain type A spermatogonia only. It has therefore been proposed that some germ cell types present in the seminiferous epithelium between stages X-VI have an inhibitory effect on Sertoli cell GATA-1 expression. Stages VII-VIII are androgen

dependent therefore it has also been suggested that expression of GATA-1 could also be regulated by androgens.

1.5.8.5.2. GATA-4

Abundant GATA-4 mRNA transcripts are observed in undifferentiated gonads of mouse embryos on fetal day 11.5 (Viger *et al.*, 1998). By fetal day 13.5 GATA-4 mRNA expression is restricted to somatic cells of both testes and ovaries. A day later GATA-4 expression becomes sexually dimorphic with abundant levels of GATA-4 mRNA maintained in early Sertoli cells and mesenchymal Leydig cell progenitors. However expression of GATA-4 is rapidly downregulated in the ovary and does not return to the ovaries until full maturity is reached. Expression of GATA-4 mRNA in neonatal Sertoli and Leydig cells is maximal between birth and postnatal day 14, after which mRNA levels decrease with increasing age but are still detectable in adult testes (Ketola *et al.*, 1999). GATA-4 protein levels increase during neonatal development and reach a peak between day 4 (Ketola *et al.*, 1999) and day 7 (Viger *et al.*, 1998). In agreement with mRNA levels, GATA-4 protein expression then decreases with increasing age. There is some disagreement concerning the cellular location of GATA-4 expression in the adult testes. Heikinheimo et al., (1997) and Ketola et al., (1999) state that GATA-4 expression is restricted to somatic cells of the adult testis and ovary. In contrast, Viger et al., (1998) observed a switch in GATA-4 protein expression from Sertoli cell to germ cells after day 23 resulting in germ cell specific expression of GATA-4 in the adult testis. This is in agreement with Arceci et al., (1993). It was proposed that loss of GATA-4 expression from Sertoli cells coincides with the observed increase in Sertoli cell GATA-1 expression. It was suggested that GATA-1 takes over the role of GATA-4 in the mature Sertoli cell (Viger *et al.*, 1998). The sexually dimorphic expression of GATA-4 observed during gonadal embryonic development is associated with the earlier onset of endocrine function in the testes when compared to the ovaries. Maximal levels of GATA-4 in Sertoli cells are concurrent with the proliferative phase of Sertoli cell development. Therefore it has been suggested that GATA-4 may play a role during Sertoli cell mitosis and Leydig and Sertoli cell endocrine function.

Administration of FSH to a mouse Sertoli cell line MSC-1 and a Leydig cell tumour cell line resulted in a modest increase and a significant increase in GATA-4 mRNA levels, respectively (Ketola *et al.*, 1999). Similar results were obtained by Heikinheimo et al., (1997) following treatment of MSC-1 Sertoli cells and a granulosa cell line with FSH and forskolin. In vivo experiments have indicated that testosterone is not required for GATA-4 expression in Sertoli cells. Normal levels of GATA-4 were observed in hypogonadal mouse testes (Ketola *et al.*, 1999). In addition treatment of mice with the GnRH antagonist azaline b did not alter GATA-4 protein levels and 15 days after EDS treatment there was no difference in levels of GATA-4 protein between control and treated animals (Ketola *et al.*, 1999). In contrast hCG stimulates an increase in GATA-4 expression by Leydig cells. A reduction in Leydig cell LH receptor levels is associated with a decrease in GATA-4 mRNA levels. Thus FSH and LH stimulate GATA-4 expression by Sertoli cells and Leydig cells respectively.

GATA-4 expression in immature mouse granulosa cells can be upregulated after four days of DES treatment, GATA-4 levels decline after two days of DES withdrawal and GATA-4 expression is undetectable following DES withdrawal and administration of testosterone (Heikinheimo *et al.*, 1997). It must be noted that following DES withdrawal there is a dramatic increase in the number of apoptotic cells within the follicles which become severely atretic following DES withdrawal and testosterone administration. Therefore it is a possibility that GATA-4 expression by immature granulosa cells can be induced by oestrogen treatment. However GATA-4 may just act as a survival or mitotic factor during follicular development in the ovaries.

Two GATA motifs have been located at positions –147 bp and –114 bp within the inhibin-α promoter region. Using cotransfection and expression experiments in an MA10 Leydig tumour cell line Feng et al., (1998) have shown a dose dependent GATA-1 induced upregulation of inhibin-α expression. GATA-4 has no effect on the activity of the inhibin α promoter in the cotransfection experiments but is able to cause a specific band shift in EMSA experiments. Ketola et al. (1999) have questioned this finding due to the fact that GATA-1 is not normally expressed in Leydig cells whereas GATA-4 is. In vitro transactivation experiments have indicated that GATA-4 can induce a

significant upregulation in inhibin-α expression in Leydig tumour and granulosa cell lines. The importance of these findings is supported by the temporal expression pattern of GATA-4 in the testis. GATA-4 expression is switched on earlier than that of both inhibin subunits and GATA-4 is expressed in both Leydig and Sertoli cells. This evidence suggests that GATA-4 is able to induce inhibin-α expression in both Leydig and granulosa cell lines. In addition it is also possible that both GATA-4 and GATA-1 could transactivate inhibin-α expression in Sertoli cells in vivo.

The StAR promoter also contains a GATA motif within its functional promoter region. Silverman et al., (1999) have shown that the conserved GATA binding motif is essential for StAR expression in prepubertal rat granulosa cells. Mutation of the binding site results in a decrease in promoter activity. GATA-4 is expressed in Leydig cells prior to the onset of testosterone secretion and GATA-4 expression is thought to be upregulated by LH. Therefore it is possible that GATA-4 also has a role to play in steroidogenesis in the Leydig cell.

1.5.8.6. Mouse-Musashi-1

Mouse-Musashi-1 (Msi1) is a 39kDa RNA binding protein (RBP) and is a member of the Musashi subfamily of RBPs which include drosophila Musashi-1 (*d-Msi*), *Xenopus laevis* nervous system-specific RNP protein-1 (*Nrp-1*) and the human homologue Musashi-1 (MSI1). All four members of the family have a conserved structure made up of two conserved tandem RNA recognition motifs (RRM) which contain short, highly conserved regions called RNP1 (octamer) and RNP2 (hexamer). The RRM sequences of *d-Msi* and Nrp-1 are 77% and 90% homologous to Msi1 RRM sequences, respectively. Members of the Musashi family have a conserved expression pattern which was originally thought to be restricted to the nuclei of CNS stem cells and neural precursor cells. Expression is always greatly downregulated in fully differentiated and postmitotic neurones and glial cells. The restricted expression pattern of Musashi family members to nuclei of neurone cells, suggested that Msi1 was involved in RNA processing, a role carried out by other RBPs which are most homologous to Msi1.

In addition to expression of Msi1 transcripts in fetal and adult neural tissue, Northern blot analysis has shown expression of a 3.1kb transcript in the small intestine and the ovary (Good *et al.*, 1998). Msi1 expression in the ovary was thought to be related to maternal expression similar to that observed in *d-msi*. However Maguire et al.,(1999) located Msi1 and a rat homologue to Sertoli cells in mouse and rat testis. Using both in situ hybridisation and immunocytochemistry, Msi1 expression is restricted to Sertoli cells and is not seen in any other cell type within the testis. Expression is highest in Sertoli cells from immature testis (day 6) and is shown to decrease with increasing age. In contrast to Msi1 expression in neuronal cells where expression is restricted to the nuclei, Msi1 protein expression is observed in both nuclei and cytoplasm of fetal and immature rodent Sertoli cells. Once Sertoli cells had ceased dividing (around day 14-21) expression becomes predominantly nuclear. This indicated that Msi1 plays an additional or alternative role to RNA processing in the neonatal Sertoli cell. Msi1 may be involved in nuclear-cytoplasmic RNA trafficking in mitotic Sertoli cells. However during Sertoli cell maturation Msi1 then became predominantly involved in RNA processing within the nucleus. In the adult testis Msi1 expression is seen in Sertoli cell nuclei at all spermatogenic stages. However expression is most abundant in tubules at stages XI-VI which corresponds to a time when testosterone levels are at their lowest. It has therefore been suggested that Msi1 may be negatively regulated by testosterone (Maguire *et al.*, 1999).

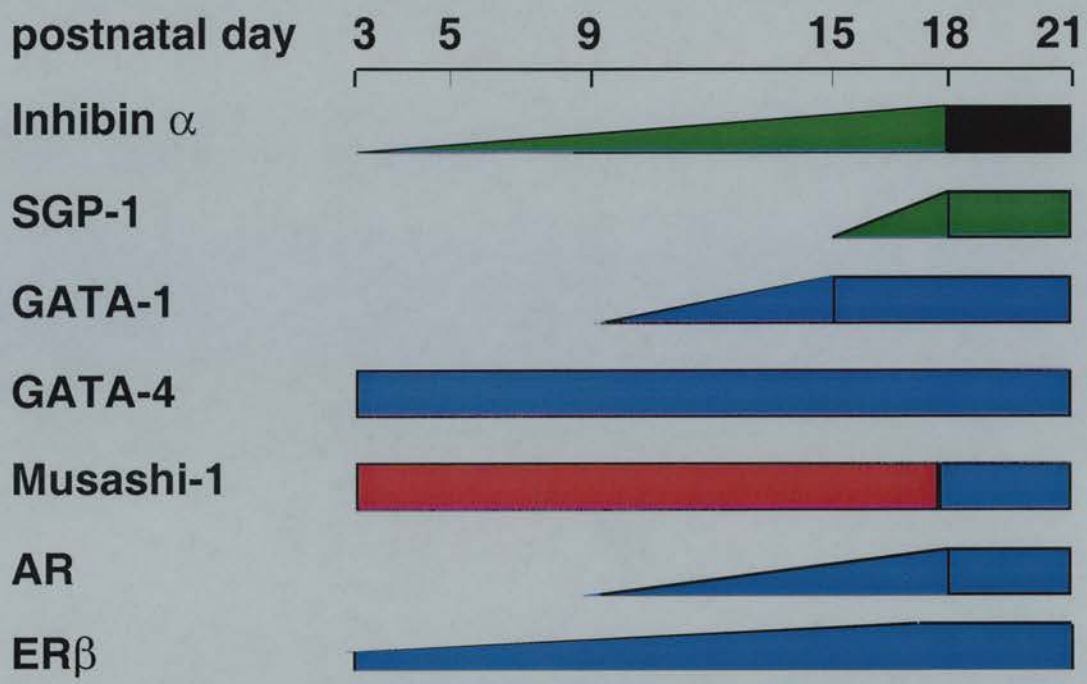

Figure 1.6: Diagram to show changes in Sertoli cell protein expression patterns during early postnatal development. Sertoli cell cytoplasmic staining is green; stage specific cytoplasmic staining is shown in black; nuclear Sertoli cell immunostaining is shown in blue and joint cytoplasmic and nuclear staining is represented by red.

1.6. Influence of Oestrogenic Chemicals on Postnatal Gonadal Development

Over the past nine years, an increasing number of papers in the literature have expressed concerns that human male reproductive health is declining and that the incidence of congenital abnormalities and cancers of the male reproductive tract is increasing. The causes behind these reported increases are not known but one hypothesis that has been promoted in both the scientific community and the media is that exposure to exogenous oestrogen or oestrogen like compounds (xenoestrogens and environmental oestrogens) may be involved.

1.6.1. Sources of Oestrogenic Chemicals in the Environment

Currently there are four major classes of environmental hormones that interact directly with steroid hormone receptors: environmental oestrogens,

environmental antioestrogens, environmental antiprogestins and environmental antiandrogens. Environmental oestrogens include phytoestrogens and synthetic chemicals such as pesticides, herbicides, bisphenol A, some polychlorinated biphenyls (PCBs), plasticisers (phthalates) and surfactant breakdown products also known as alkylphenol polyethoxylates (APEOs). During sewage treatment APEOs are broken down by microbes and some of the degradation substances produced have oestrogenic activity (octyphenol and nonylphenol) (Turner and Sharpe, 1997). Dioxins are known to have antioestrogenic activity and polyaromatic hydrocarbons, linuron, vinclozolin and *p,p'*- DDE have antiandrogenic activity (for review see (Cheek and McLachlan, 1998).

All the synthetic environmental oestrogens described are at least 1000 times less potent than oestradiol (Arnold *et al.*, 1996). Exposure to very high levels of environmental oestrogens would therefore be required to cause any biological effect. However these chemicals may be capable of causing additive, synergistic or antagonistic effects. A recent study which examined sons of farmers and gardeners for abnormalities in the genital tract found a significant increase in the risk of cryptorchidism but not hypospadias in the sons of mothers (but not fathers) who worked in these occupations. Farmers and gardeners use a number of chemicals which are known to be oestrogenic or possess other hormone disrupting effects. Thus occupational exposure to certain environmental endocrine disrupters can be high enough to cause an increased risk of congenital abnormalities (Weidner *et al.*, 1998).

Natural plant or phytoestrogens are more likely to be a significant source of human oestrogen exposure than synthetic oestrogens. Plant oestrogens are found in soya, grains, vegetables and fruits and therefore humans consume a large quantity of phytoestrogens in their diet. A high dietary intake of phytoestrogens causes reproductive abnormalities in sheep (Kaldas and Hughes, 1989) and can prolong the follicular phase of the menstrual cycle in humans (Cassidy *et al.*, 1994).

1.6.2. Evidence of Oestrogenic Effects on Male Fertility

In male rodents it is well established that exposure to supra physiological doses of oestrogens during the critical period of neonatal differentiation results in an array of permanent reproductive defects, which include a decline in sperm counts (de Mouzon *et al.*, 1996), atrophy of testes and sexual accessory glands (Brown-Grant *et al.*, 1975; Kincl *et al.*, 1965; Newbold and McLachlan, 1985), impaired maturation of germ, Sertoli and Leydig cells (Gaytan *et al.*, 1986; Pinilla *et al.*, 1992; Sharpe *et al.*, 1998), altered LH secretion (Pinilla *et al.*, 1995) and inappropriate puberty onset (Bellido *et al.*, 1990; Brown-Grant *et al.*, 1975). Disruption of reproductive function by neonatal oestrogenisation is proposed to arise via numerous mechanisms and the elucidation of such mechanisms is still being researched.

It was first proposed in the early 1990s that exogenous oestrogens in the environment were affecting sperm counts. In 1992 (Carlsen *et al.*, 1992) analysed data from 61 studies spanning 50 years and including approximately 15,000 men. The data suggested that a decrease in both sperm concentration and semen volume had occurred over time. Sperm concentrations had fallen from an average of 113 million/ml to 66 million/ml and semen volume was reduced from 3.4 ml to 2.75 ml. The validity of the conclusions drawn from this study have been questioned. However similar studies that were carried out in the 1970s and 1980s also suggest that sperm counts have fallen and a negative correlation between later year of publication and lower average sperm production was observed (James, 1980).

Following these initial studies numerous other papers using various methods of data analysis and different groups of men have been published. Studies carried out in Paris, Scotland and Belgium (Auger *et al.*, 1995; Irvine *et al.*, 1996; van Waelegham *et al.*, 1996) stated that semen quality was falling and that a relationship existed between sperm count and year of birth. Other studies in France and Finland reported that sperm concentrations had not changed (Bujan *et al.*, 1996; Suominen and Vierula, 1993) whilst studies in the USA found a small but significant increase in sperm counts had occurred between 1970 and 1994 (Fisch *et al.*, 1996). The largest retrospective review of semen quality was carried out on 7714 men and reported a significant decrease in semen quality

with later year of birth; the mean sperm concentration of men born before 1939 was 92.5 million/ml compared to 77.1 million/ml for men born after 1965 (de Mouzon *et al.*, 1996).

Thus if sperm concentrations were falling the literature indicates that it is a regional phenomena which supports the hypothesis that factors in the local environment are modifying male reproductive health. The data in the above studies are inconclusive and all studies have been criticised as they are retrospective and comparisons across studies can not be carried out due to differences in selection criteria and methods of collection and analysis.

Other strong indicators of problems with male reproductive health are the observed increases in the incidence of testicular germ cell cancer and congenital abnormalities of the male reproductive tract. Incidences of testicular germ cell cancer have been steadily increasing world wide, over the last 30 years such that testicular cancer is now the commonest malignancy of men aged 15-45 in the UK. The cause(s) of testicular cancer are unknown but it is proposed that events occurring in utero during testis development lead to persistence of fetal gonocytes within the testis which lead to the formation of pre-invasive carcinoma in situ cells (CIS) (Skakkebaek *et al.*, 1987). It has been hypothesised that these cells become invasive after increased exposure to hormones which occurs at puberty (Cortes *et al.*, 1987).

There are several reports of increasing incidences in congenital abnormalities of the male reproductive tract. Cryptorchidism is a condition when the testicles do not fully descend into the scrotal sac but remain within the abdomen. In Scotland, England and Wales studies show an increase in the number of boys undergoing surgery to lower the testis over the last 40 years (Campbell *et al.*, 1987; Chilvers *et al.*, 1984). Hypospadias is a congenital abnormality of the penis in which the urethral opening does not appear at the tip of the glans but can occur anywhere along the shaft of the penis. A number of studies carried out across the world have reported an increase in the incidence of hypospadias. However a review of data collected from a number of different countries does not indicate an increase in hypospadia occurring over time but that increases in

the abnormality are more frequently reported in more affluent nations (Paulozzi, 1999).

Male alligators in Lake Apopka, Florida had abnormal germ cells and abnormally small phalli following a bad chemical spill in the lake involving trichloroethane (DDT) which can be degraded into DDD and *p, p* 'DDE which have oestrogenic and anti androgenic effects, respectively. Normal male sexual development of the juvenile male alligators had been permanently modified by exposure to high doses of the polluting chemicals (Guillette *et al.*, 1994). Delayed maturation, smaller gonads, a lack of secondary sex characteristics, reduced serum testosterone and dysfunctional hypothalamic-pituitary-gonadal axis have all been observed in many species of fish living in water downstream from kraft pulp mills (e.g. white sucker). Kraft mill effluents usually contain high levels of dioxin and phytoestrogen β-sitosterol (Munkittrick *et al.*, 1991). In addition rainbow trout living downstream from sewage treatment outflows have increased incidence of hermaphroditism and produce large amounts of the egg protein vitellogenin (Jobling and Sumpter, 1993; Purdom *et al.*, 1994). Alkylphenol-polyethoxylates are a major group of surfactants found in sewage and are known to be oestrogenic to fish (Jobling *et al.*, 1996). A study of ejaculate volumes from Florida panthers found low sperm counts, low ejaculate volumes and a high proportion of abnormal sperm. The incidence of cryptorchidism had also increased. It was proposed that these abnormalities were due to the bioaccumulation of *p,p'*-DDE and PCBs in the body fat of the panthers as these chemicals have antiandrogenic and oestrogenic activity, respectively, (Facemire *et al.*, 1995). However it has also been argued that the described abnormalities in Florida panthers could also result from in breeding.

In most cases there is no definitive proof that the changes observed in wildlife have been caused directly by exposure to environmental oestrogens. When the causative agent is known, the effects on the reproductive tract have been induced only after exposure to high levels of environmental chemical. Thus when discussing the observed effects of environmental oestrogens on wildlife it is necessary not to extrapolate the causes directly to the reported changes in human male reproductive health.

The synthetic oestrogen diethylstilbestrol (DES) was prescribed to pregnant women between 1940s to 1970s to prevent miscarriages. The sons of mothers exposed to DES during pregnancy had increased incidence of hypospadias, epididymal cysts, hypoplastic testis, cryptorchidism, microphallus and abnormal semen parameters (Gill *et al.*, 1977; Gill *et al.*, 1979). In addition, sperm counts were significantly lower in some DES exposed men (Gill *et al.*, 1979) and there is some evidence to suggest that DES induced a small increase in the incidence of testicular cancer (Vessey, 1989).

Researchers have linked the above effects on male reproductive tracts to endocrine disrupting chemicals in the environment. However exactly how such chemicals interact with each other to counteract or synergise with endogenous pathways within biological systems is not known.

1.6.3. Possible Mechanisms of Oestrogenic Action During Postnatal Testis Development

Despite the classical contention that oestrogen is the 'female hormone', reports so far indicate that oestrogen plays a pivotal role in the regulation of development and function in several systems in the male. In mammals these actions include permanent masculinisation of brain structures involved in neuroendocrine control of gonadal function and reproductive behaviour (Arnold and Gorski, 1984), regulation of differentiation and function of Leydig cells in the neonatal testis (Abney and Myers, 1991; Brinkman *et al.*, 1980), modulation of luminal fluid resorbtion in the epididymis (Hess *et al.*, 1997) and control of FSH secretion (Sharpe *et al.*, 1998) as well as direct effects on bone and the cardiovascular systems (Sharpe *et al.*, 1998).

The mechanisms by which inappropriate levels of oestrogen could cause the abnormal development of the male reproductive tract are unknown. Neonatal administration of oestradiol benzoate to male rats causes a significant dose dependent delay in spermatogenesis thought to result from distension of the rete testis and backflow impairment (Aceitero *et al.*, 1998). This has also been observed following neonatal administration of DES to rats. Resorbtion of STF in the excurrent ducts was defective and this led to distension of the rete testis (Fisher *et al.*, 1998b). Infertility in male ERKO mice is partly caused by

impairment of STF resorbtion in the efferent ducts and subsequently the rete testis are distended and spermatogenesis is disrupted (Hess *et al.*, 1997). These findings appear to contradict the role of oestrogen in STF resorbtion and therefore highlight the complexities involved in establishing the role of oestrogen in male reproductive development and the mechanisms by which disruption in male development can arise.

Fertility in the male can also be compromised by alteration in germ cell, Leydig cell and Sertoli cell function through direct action of oestrogen on ERα and ERβ. Administration of oestradiol to adult rats increases germ cell apoptosis in patterns distinct from those observed following gonadotropin and testosterone withdrawal (Atanassova *et al.*, 1999; Blanco-Rodriguez and Martinez-Garcia, 1997). However administration of low doses of DES can also advance spermatogenesis demonstrated by increases in the volume of spermatocytes per Sertoli cell following DES treatment (Atanassova *et al.*, 1999). Thus oestrogen may play a role in regulating early germ cell development by direct action on spermatocytes and spermatogonia through expression of ERβ (Saunders *et al.*, 1998).

Oestradiol can also alter fertility through direct and paracrine effects on Leydig cell differentiation, number and steroidogenesis. Oestradiol produced by aromatisation of testosterone in the immature Sertoli cell, blocks ontogenic development of adult type Leydig cells from precursor cells in the neonatal testis (Abney, 1999). It is also postulated that oestradiol acts directly on mature Leydig cells to block androgen production by inhibiting the activities of certain steroidogenic enzymes (Abney, 1999) and decreasing AR levels (Sharpe *et al.*, 1998; Tena-Sempere *et al.*, 2000) thus inhibiting testosterone production (Atanassova *et al.*, 1999) and androgen action in the developing testis.

Neonatal administration of oestradiol can also impair spermatogenesis and male reproductive development through modification of Sertoli cell maturation and function. It was originally proposed that oestrogen treatment during fetal and prepubertal development indirectly altered male reproductive health through suppression of gonadotropin secretion by the anterior pituitary. Decreased levels of FSH and LH led to decreased Sertoli cell numbers resulting

in lowered sperm output in the adult testis, delayed onset of puberty and impaired spermatogenesis due to permanent modification of Sertoli cell function (Gaytan *et al.*, 1986). Recent studies have shown that most of the effects of neonatal oestrogen administration cannot simply be explained by suppression of gonadotropin levels (Atanassova *et al.*, 1999; Sharpe *et al.*, 1998). Neonatal treatment of rats with varying doses of DES and ethinyl oestradiol causes a reduction in Sertoli cell numbers, a delay in normal expansion of spermatogenesis and an increase in abnormal sperm as well as permanent impairment of Sertoli cell functional maturation, demonstrated by a change in expression levels of AR in the adult rat Sertoli cell. These results suggest that oestrogen plays a physiological role in the maturational development of Sertoli cells (Atanassova *et al.*, 1999; Sharpe *et al.*, 1998).

Neonatal immature Sertoli cells not only express ERβ but are also a source of oestrogen in the prepubertal testis and oestrogen can therefore exert autocrine effects on Sertoli cells during development. Oestrogen upregulates ERβ mRNA levels and TGFβ levels in neonatal Sertoli cells (Dorrington and Khan, 1993; Tena-Sempere *et al.*, 2000). TGFβ and ERβ in association with FSH act to increase Sertoli cell mitosis. Sertoli cell mitotic activity in the prepubertal testis is proposed to be inhibited during normal testis maturation by paracrine factors such that Sertoli cell proliferation ceases and cell differentiation begins. However following DES or environmental oestrogen exposure such control mechanisms regulating Sertoli cell proliferation will be short circuited and consequently the artificially elevated oestrogen levels will result in a delay in Sertoli cell maturation.

Sertoli cells carry out crucial functions in the postnatal development and differentiation of Leydig cells and germ cells through paracrine interactions in the prepubertal testis. Modification of Sertoli cell function and maturation by increased oestrogen levels can modify Leydig cell differentiation and delay the onset of spermatogenesis. Spermatogenesis is also affected by a decrease in testosterone levels which occurs in the adult rat testis following neonatal administration of DES (Atanassova *et al.*, 1999). Sertoli cell maturation is a prerequisite for normal spermatogenesis and permanent impairment of Sertoli

cell functional maturation will therefore contribute to spermatogenic damage in the mature testis of treated animals (Gaytan *et al.*, 1986).

AR and ERs are all expressed at a number of cellular sites where aromatase is also expressed. It is therefore a possibility that the balance between oestrogen and androgen action is finely regulated at the local level. Androgens regulate aromatase expression levels whilst oestrogen can inhibit testosterone synthesis and AR expression. Oestrogen can also bind to AR and alter transactivation of androgen regulated genes (Yeh *et al.*, 1998) as well as inhibiting transcriptional activation of AR when bound to ER (Kumar *et al.*, 1994). Administration of either antiandrogens or potent oestrogens causes remarkably similar changes in the male reproductive tract which suggests that common pathways of action are involved in these changes (Sharpe, 1998). Antiandrogens may elevate endogenous oestrogen levels which cause additional changes to male development on top of straightforward effects caused by a blockade in androgen action. Conversely administration of oestrogen will interfere with testosterone production and androgen action in addition to activating ER mediated pathways. Both these scenarios would result in increased oestrogen action at the expense of reduced androgen action. Thus exposure to inappropriate levels of oestrogen during postnatal life could be modifying male reproductive development by changing the testosterone:oestrogen balance which will in turn permanently affect Sertoli and Leydig cell maturation and function.

1.7. Aims of this study

The aims of this study were to investigate effects of oestrogen and testosterone administration on immature rat Sertoli cell maturation. Initially, expression patterns of known and novel markers of Sertoli cell functional maturation were determined during normal postnatal development of the rat testis, after which the effect of oestrogen and testosterone treatment on the expression patterns of these factors was investigated at both protein and mRNA levels. In order to clarify if changes in gene expression by steroids were the result of direct or indirect actions on Sertoli cells, primary rat Sertoli cell cultures and an immortalised mouse Sertoli cell line were included in this study.

Chapter 2.

General Materials and Methods

The techniques outlined in this chapter are common to a number of studies in this thesis. Methods specific to individual experiments are described in the relevant chapters.

2.1. Chemicals and suppliers

Molecular biology grade chemicals were obtained from Sigma, (Poole, UK) and IBI, (Cambridge, UK). All radiolabelled nucleotides were obtained from Amersham, (Buckinghamshire, U.K.). Enzymes were purchased from Boehringer Mannheim, (Lewes, UK) or Promega, (Southampton, UK). Phenol/chloroform was purchased from CAMLAB, (Cambridge, UK) and was pre-buffered with Tris. pH 8.0. Autoradiography products were obtained from Eastman Kodak, (Rochester, NY, USA), supplied by Sigma.

2.2. Animals

2.2.1. Rats

Animals used for these studies were mainly male Wistar rats; immature, aged post partum day 3-35 and adult, aged day 65-90, when day of birth is called postnatal day 1. The rats were bred in the MRC Reproductive Biology Unit in Edinburgh and were maintained in standard conditions of a 12 hours light:12 hours dark cycle and an ambient temperature of 21°C. Food and water were available *ad libitum.* Animals were killed by asphyxiation with CO_2 followed by cervical dislocation.

2.2.2. Mice

Mice used in these studies were male C57black x CBL mice; immature, aged post partum day 1-16, adult, day 40 and older when day of birth is designated as postnatal day 1. The mice were bred in the MRC Reproductive Biology Unit in Edinburgh and were maintained under standard conditions of 14 hours light: 10 hours dark cycle and at an ambient temperature of 21°C. Food and water

dissolved in RNase-free water by warming to 65°C for 5 minutes. RNA isolated in this way was then stored at -70°C.

RNA was scanned at 260 and 280nm on a spectrophotometer (GeneQuant, Pharmacia Biotech, Cambridge, UK). The 260:280 ratio for each sample was calculated to give an estimation of the purity of the RNA. A ratio of 1.6-1.9 was considered acceptable. The concentration of the RNA was calculated from the 260nm value where an optical density of 1.0 is equal to 40μg/ml RNA.

2.3.3. Separation of RNA on denaturing agarose gels

RNA was separated on a 1.5% denaturing agarose gel for both assessment of RNA quality and prior to blotting onto nylon membranes for Northern analysis (section 2.9). The gel was prepared by melting 2.25g Seakem agarose (Boehringer Mannheim, Mannheim, Germany) in 127.5ml pure water. This was cooled to about 60°C and 15ml of 10x running buffer (containing 200mM MOPS, 10mM EDTA and 50mM sodium acetate at pH 7.0) plus 8.1ml 37% formaldehyde was added. The solution was mixed gently and poured into a gel tray (15 x 20cm) containing a 15 or 20 well comb in a fume hood. After setting, the comb was removed and the gel submerged in 1x running buffer in a Sub-Cell electrophoresis cell (BioRad Laboratories, Hemel Hempstead, U.K.).

RNA (up to 20μg) was prepared by adding 16μl sample buffer and heating at 60°C for 5 minutes. Sample buffer contained 100μl 10x running buffer, 500μl deionised formamide and 178μl formaldehyde. After heating, 8μl dye solution containing 7.5% w/v ficoll 400, 0.1% w/v bromophenol blue and 1μl of 1mg/ml ethidium bromide was added to each sample. RNA was loaded into individual wells of the gel and separated by running overnight at 34V or for 5 hours at 120V.

2.4. Tissue fixation and processing

2.4.1. Tissue fixation

Testes were quickly removed from asphyxiated rats, decapsulated and weighed before they were placed in Bouin's fluid for 5 hours. Testes removed from rats

aged day 35 and adults were perfusion fixed in Bouin's fluid by Dr. Richard Sharpe. Perfusion fixed testis were then cut into 2-3mm transverse slices. Fixed tissue was transferred to 70% ethanol for storage until processing for immunohistochemistry.

2.4.2. Processing and sectioning of tissue

Tissue was processed through a graded series of alcohols in an automatic TP1050 Processor (Leica, Milton Keynes, UK) using a standard 20 hour cycle and embedded in paraffin wax. Tissue processing was kindly performed by Mr. Mike Millar and Mrs Sheila MacPherson.

Glass microscope slides were dipped twice in a 4% v/v solution of 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone (BDH) then rinsed in acetone followed by sterile water and dried. The slides were coated to enhance the adherence of tissue sections.

Paraffin wax embedded tissue was sectioned to a thickness of 5μm using a hand operated microtome (Jung RM2035; Leica) and a D-profile knife. Sections were floated onto distilled water, transferred onto the TESPA coated slides and dried overnight before use.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

2.5.1. Oligonucleotide primers

Oligonucleotides (17-24mers) were ordered from Genosys (Exeter, UK) and were resuspended in either TE buffer (Appendix 1) or double distilled water filtered through a 6.22μm bacterial filter. The concentration of oligonucleotide was assessed by spectrophotometry at 260nm where an optical density of 1.0 is equal to a concentration of 20μg/ml.

2.5.2. RT-PCR Reaction

Partial cDNAs were generated using a combination of reverse transcription and polymerase chain reaction (RT-PCR). Reverse transcription and the production of first strand cDNA pools was undertaken using 1μg of total RNA and either

100ng/μg RNA of random primers (Promega) or 500ng/μg RNA of oligo dT (12-18) primers (Gibco-BRL) in a volume of 10μl made up with distilled water. Oligo dT primers reverse transcribe mRNA species possessing a polyA sequence at their 3' end. Primers and RNA were denaturated at 70°C for 10 minutes followed by immediate annealing on ice for 5 minutes. First strand buffer (50mM Tris-HCl pH 8.3, 75mM KCl, 5mM $MgCl_2$), 10mM DTT (both from Gibco BRL) and 500μM of each dNTP (Amersham Pharmacia Biotechnology, St. Albans, UK) was added to each reaction, mixed and incubated at 37°C for 1 minute. 200 U of Superscript™ II RNase H⁻ Reverse Transcriptase (Gibco BRL) was then added to the reaction mixture. Oligo dT primed reactions were incubated at 37°C for 1 hour and random primed reactions were incubated at 21°C for 10 minutes followed by 50 minute incubation at 37°C. The reverse transcription reaction was terminated by the addition of 8mM EDTA pH 8.0 and cDNA pools were stored at –20°C.

Double stranded DNA was amplified by polymerase chain reaction (PCR;(Saiki *et al.*, 1988) using the first strand cDNA as template. Specific 5′ and 3′ oligonucleotide primers were used at a final concentration of 0.5μM in PCR reactions containing 100μM of each dNTP (Amersham Pharmacia Biotechnology), Taq polymerase buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100) and 2.5U Taq polymerase (P.E. Applied Biosystems, Warrington, UK). Template cDNA was used at a 1:50 dilution. Thirty-five cycles of amplification were performed with annealing temperatures determined by the melting temperature (Tm) of the primers used. The Tm is dependent on the nucleotide content of the primers and was calculated using the formula (4 x G+C) + (2 x A+T). Extension occurred at 72°C and lasted 35 seconds to 2 minutes dependent on the length of desired template.

2.5.3. Cloning of amplified cDNAs

Where RT-PCR reactions were conducted for obtaining probes for RNase protection assay reactions, the amplified DNA fragments were subcloned into appropriate plasmid vectors containing SP6 and T7 RNA polymerase

promoters. The vectors used were the TA cloning vectors, pCRII (Invitrogen, Abingdon, UK) illustrated in Figure 2.1. and pGEM-T easy vector, illustrated in Figure 2.2. (Promega). Amplification of the cDNA was followed by ligation of the PCR product and plasmid.

The pCRII vector was ligated to the PCR product using the TOPO™TA cloning® kit (Invitrogen) and was carried out following manufacturer's instructions. The vector is provided already linearised and covalently bound to topoisomerase which undergoes spontaneous ligation after 5 minutes incubation at room temperature. 5-20ng of PCR product and 1 volume of vector were mixed together to give a final reaction mix of 5µl made up with water.

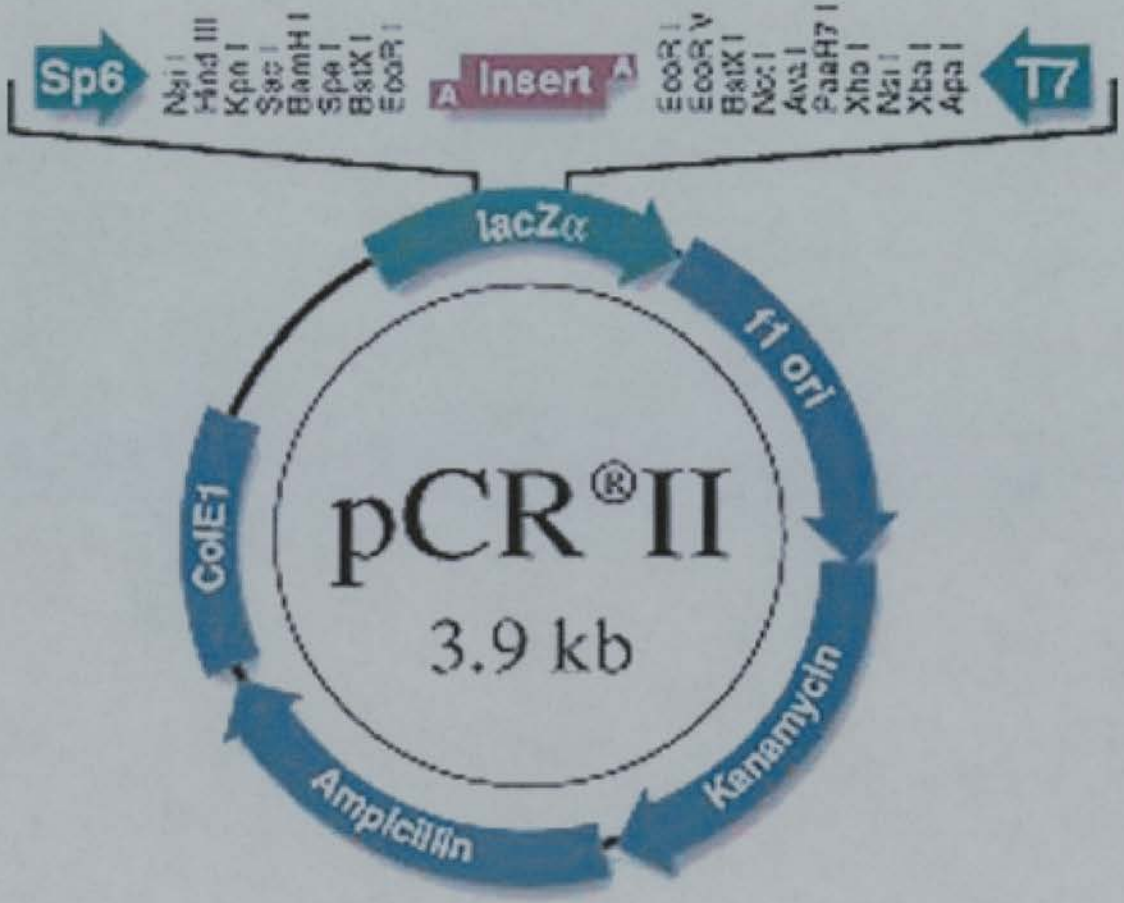

Figure 2.1: Map of the pCR® II vector. The pCR®II vector shown above was used in the subclonng of RTPCR products. The PCR product was ligated into the region marked 'insert' using TA cloning such that the insert was flanked on either side by *EcoRI* restriction sites. Diagram adapted from Invitrogen web site.

The pGEM-T easy vector was ligated with the PCR product at appropriate molar ratios (3:1 respectively) with T4 DNA ligase (Promega). The concentration of PCR product was assessed by comparison with known standards on an agarose gel and was added to the ligation mixture such that there was a 1:3 molar ratio of plasmid to PCR product. 25ng plasmid vector and 1U T4 DNA ligase (as supplied by plasmid supplier) were mixed with 75ng of PCR product, 1µl 10x ligation buffer (contains 300mM Tris-HCl pH 7.8, 100mM $MgCl_2$, 100mM DTT and 10mM ATP) and made up to a volume of 10µl with water.

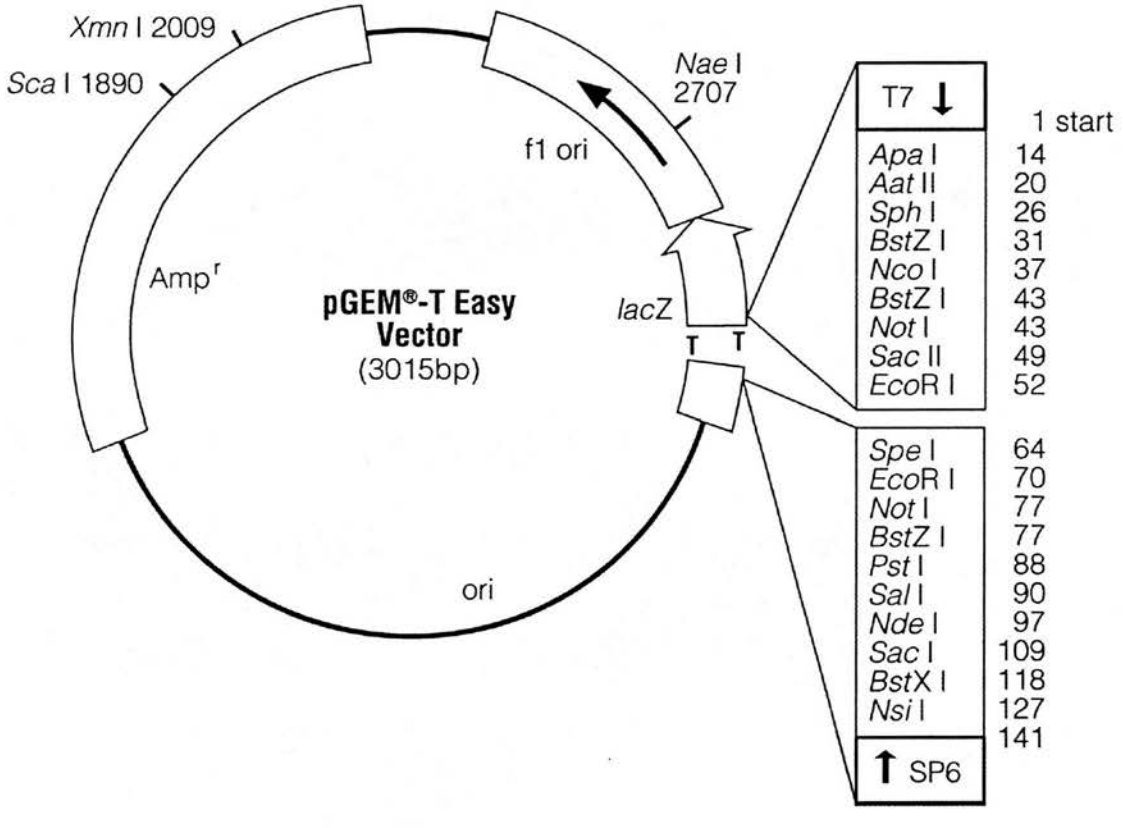

Figure 2.2: Map of pGEM ®–T Easy vector. RTPCR products were subcloned into the pGEM ®–T Easy vector using T4 DNA ligase. The PCR insert was ligated into the region between the two T's such that the insert was flanked by an *EcoRI* site and a *SpeI* site. Diagram adapted from Promega web site

Following ligation, transformation of competent TOP10F' (pCRII vector) or JM109 (pGEM-T easy vector) E.Coli cells was carried out as follows; 2µl of 0.5M β-mercaptoethanol was added to each vial of competent cells prior to the addition of 2µl of ligation reaction (as described above). The mixture was

incubated on ice for 30 minutes, heat-shocked by incubating at 42°C for 30 seconds (pCRII) or 45 seconds (pGEM-T) and then placed on ice for 2 minutes. Thereafter 450µl SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) prewarmed to 37°C was added to the cells which were subsequently incubated at 37°C on a shaker at 225rpm for 1 hour. Aliquots (50µl and 100µl) were plated onto Luria Bertani (LB) agar plates (Appendix I) containing 50µg/ml ampicillin, 50µl 50µg/ml X-gal and 100µl 100mM IPTG (pCRII) or 100µg/ml ampicillin, 50µl 50µg/ml X-gal and 100µl 100mM IPTG (pGEM-T) with a bent glass rod. Plates were incubated inverted overnight at 37°C.

Identification of bacterial colonies which were transformed with plasmid containing the cDNA insert was based on the *lac Z* blue/white selection. Both pCRII and pGEM-T easy vectors contain the *lac Z* gene in their polylinker region (Figures 2.2. and 2.1.). *Lac Z* gene expression was induced by IPTG to express the product β–galactosidase. This acted on the substrate X-gal, which β–galactosidase converted into a blue coloured end product. The *lac Z* gene was disrupted by the insertion of the cDNA sequence into the vector by ligation. Therefore bacterial colonies that contained plasmid without the cDNA insert had a functional *lac Z* gene and were blue whereas those colonies which were successfully transformed with recombinant plasmid were white.

The bacterial colonies containing the cloned plasmid were restreaked on LB agar plates which were again incubated inverted overnight at 37°C. Individual white bacterial colonies were picked into LB broth (Appendix I) and were propagated overnight at 37°C in LB broth containing 50µg/ml ampicillin in a shaker at 225 rpm.

2.6. Plasmid recovery and analysis

2.6.1. Plasmid recovery

Plasmid DNA was isolated for analysis from bacterial cultures by the alkaline lysis method using the Wizard Minipreps DNA purification system (Promega),

according to the manufacturer's instructions. Briefly, bacterial suspensions were pelleted by centrifugation at 1600g, the supernatant discarded, cells resuspended in buffer containing 50mM Tris/HCl, pH 7.5, 10mM EDTA and 100μg/ml RNase and mixed with an equal volume of 0.2M NaOH containing 1% SDS. The resulting solution containing lysed bacterial cells was neutralised by the addition of 2.25M potassium acetate. This suspension was centrifuged at 12000g to sediment the majority of bacterial genomic DNA. The supernatant (approximately 600μl) was removed, mixed with a 1ml suspension of DNA purification resin and passed through a miniprep column which retained the plasmid DNA bound to the resin. The plasmid DNA was then eluted into a sterile tube by the addition of 50μl pure water and subsequent centrifugation at 12000g for 30 seconds.

The presence of cDNA insert of appropriate size was confirmed by restriction digestion with the endonuclease EcoRI and electrophoresis of digestion products on an agarose gel (section 2.6.2.) prior to definitive analysis by DNA sequencing.

2.6.2. Analysis of plasmid DNA quality

The approximate concentration of plasmid DNA was determined by scanning at 260nm on a spectrophotometer. The concentration of the DNA was calculated from the 260nm value given that an optical density of 1.0 is equal to 50μg/ml double stranded DNA. Purity of plasmid DNA was determined by analysis on a 1.0% agarose minigel (Sambrook *et al.*, 1989) prepared using agarose (Sigma) dissolved in 1xTAE buffer (appendix I). The agarose was melted and approximately 200μg/ml ethidium bromide was added for visualisation of DNA. The gel was poured into a 7cm by 10cm gel tray containing an 8 or 12 well comb and after setting was submerged in 1xTAE buffer in 'Minnie the Gel-Cicle' cell (Hoefer, Newcastle, UK). Plasmid DNA (1μl) was run in a sample solution containing 2μl loading buffer (contained 0.25% w/v orange G (Sigma, St Louis, USA), 15% ficoll and 0.5M EDTA at pH 7.0) and 7μl water. Samples were separated in parallel with pGEM DNA markers (range 36-2645 bps, Promega) by electrophoresis at 100V for 1-2 hours in 1xTBE, viewed under UV light and photographed. Pure plasmid DNA appeared as two visible 'bands' of greater than 2kb (depending on plasmid

size); one for the circular DNA and the other representing supercoiled DNA which migrates more rapidly through the gel due to its compact form.

2.7. DNA sequencing

2.7.1. Automatic sequencing

Automatic sequencing reactions were carried out using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and were run on the Applied Biosystems Model 373A DNA sequencing system.

2.7.2. Sequencing reactions

The sequencing reaction was set up using the ABI PRISM Big Dye™ terminator cycle sequencing ready reaction kit (Perkin Elmer, California, USA). 4μl of terminator ready reaction mix (which contained Tris/HCl, pH 9.0, $MgCl_2$, thermal stable pyrophosphate, dNTPs, A-,C-,T- and I-dye terminators and AmpliTaq DNA polymerase, FS), 4μl half termmix (GENPAK Ltd, Brighton, UK) and 5 pM of primer were used for each sequencing reaction. A maximum volume of 11μl DNA template was sequenced per reaction with 30-90ng of PCR template and 200-500ng of plasmid template being optimal amounts. The total volume per reaction was adjusted to 20μl with sterile water.

Tubes were placed in a Hybaid Omn-E HBTRE thermal cycler (Hybaid, Teddington, UK) preheated to 96°C and 25 cycles of thermal cycling carried out as follows; rapid thermal ramp to 96°C, 96°C for 30 seconds, rapid thermal ramp to 50°C, 50°C for 15 seconds, rapid thermal ramp to 60°C and 60°C for 4 minutes.

On completion of the sequencing reaction, the samples were extracted with 2μl 3M sodium acetate pH 4.6 and 50μl cold 95% ethanol which were thoroughly mixed and left at 21°C for 15 minutes to remove unincorporated labelled terminators. Labelled DNA was pelleted by centrifugation at 12000g for 20 minutes, the supernatant discarded and the DNA washed using 75% ethanol, air dried and resuspended in 8μl loading buffer (deionised formamide/50mM EDTA pH 8.0 buffer (5:1)) just before loading onto the gel.

2.7.3. Automatic sequencing gel

A sequencing gel mix containing 50g urea (Sigma), 15ml 40% acrylamide and water made up to a final volume of 80ml was prepared. Amberlite resin (Sigma) was added to remove acrylamide free acid and the mixture was stirred and heated until the urea had dissolved, the solution was filtered through a 150ml filter unit (Narseve, New York, USA) and 10ml 10x TBE added. The gel mix was polymerised using 45μl TEMED and 500μl 10% ammonium persulphate and poured carefully between clean glass plates and a 0.8mm 24 well comb was used to form the lane wells.

The gel was prerun for 30 minutes at 30W. Samples resuspended in 8μl of loading buffer (section 2.8.2) were heated to 95°C for 5 minutes before loading onto the gel. The gel was run overnight using automatic data collection and analysis programs and GeneJockeyII (Biosoft, Cambridge, U.K.).

2.8. Preparation of radiolabelled probes for Northern blot analysis

2.8.1. Preparation of template DNA for radiolabelling

Double stranded DNA was usually prepared for radiolabelling by amplification of the cloned cDNA insert from a plasmid vector using a PCR mix as described in section 2.5.2. The appropriate oligonucleotide 5′ and 3′ primers used in the original amplification of the cDNA insert were used. 0.5μM of primers were used/ 50μl reactions containing 100-500ng of plasmid DNA as template. Thirty-five cycles of amplification were performed as described in section 2.5.2.

Amplification of the required DNA insert was checked by running 10μl of the PCR sample on a 1% agarose gel with known DNA markers (see section 2.6.2.). The correct sized insert was excised from the gel and subsequently purified using the QIAquick gel extraction kit (Qiagen), following the manufacturer's instructions. Briefly, 3 volumes of buffer QG were added per 100mg of gel, buffer and gel were melted at 50°C and passed through a QIAquick column at 12000g for 1 minute. A further 0.5 volumes of buffer QG was washed through

the column to remove all traces of agarose. The column and bound DNA were washed twice with 0.5 volumes of buffer PE and dried with a final 12000g spin for 1 minute. Bound DNA was eluted in 50µl of TE buffer (Appendix 1).

The concentration of purified DNA was calculated by scanning at 260nm on spectrophotometer (GeneQuant). The concentration was determined from the 260nm value given that an optical density of 1.0 is equal to 50µg/ml DNA.

2.8.2. Radiolabelling double stranded DNA

Double stranded DNA was radiolabelled by the random primer method (Feinberg and Vogelstein, 1983) using an Amersham 'Rediprime' kit (Amersham Pharmacia Biotechnology) according to the manufacturer's instructions. DNA (25-50ng) was denatured at 98°C for 5 min and was labelled with 50µCi of ^{32}P-[α]-dCTP in a reaction containing 5µl primer solution of random hexanucleotides, 10mM each dATP, dGTP and dTTP and 5µl reaction buffer containing Tris/HCl (pH 7.5), 2-mercaptoethanol and $MgCl_2$. The reaction was catalysed by addition of 2U Klenow enzyme and incubation was for 15 minutes at 37°C. Labelled DNA was denatured with 5N NaOH (100µl), neutralised with 1M Tris HCl pH 7.6 (600µl) and 1M HCl (375µl) and added to the hybridisation mixture without further purification (section 2.9.2.).

2.9. Northern blot analysis

2.9.1. RNA transfer to membrane

RNA separated on a denaturing gel (section 2.3.3.) was transferred to a nylon membrane (Hybond NX from Amersham-Pharmacia Biotech, Buckinghamshire, UK or Magna nylon transfer membrane, MSI, Westborough, MA, USA) by capillary blotting as shown in Figure 2.3. The nylon membrane was pre-wetted with pure water followed by 10x SSC (1x SSC containing 0.15M NaCl and 0.015M sodium citrate, pH 7.0) before placing carefully on the gel. Transfer using 10x SSC was allowed to continue for a minimum of 12 hours. After transfer the position of the lanes was marked on the membrane and RNA was bound using a spectrolinker X-L 1000 UV Crosslinker (Spectrotronics Corporation, Cambridge, UK).

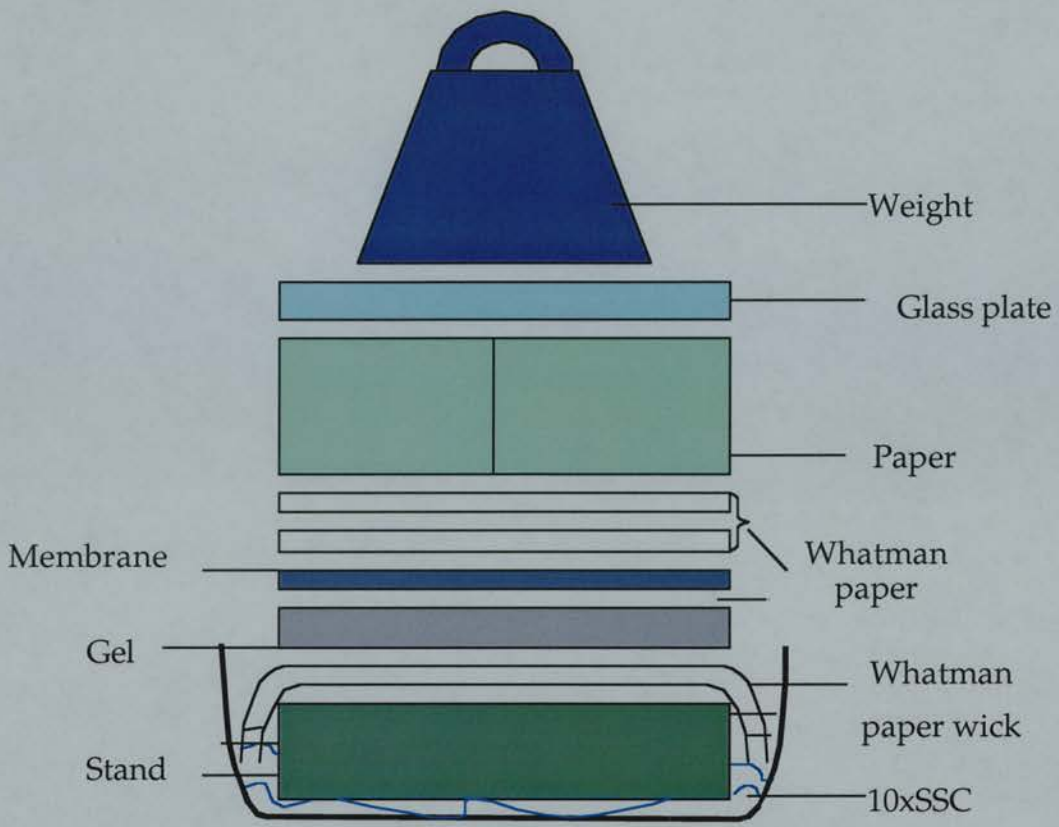

Figure 2.3. Transfer of RNA from agarose gel to nylon membrane. After total RNA had been separated by gel electrophoresis apparatus was set up as above, the gel was inverted and RNA was transferred to the nylon membrane by capillary action over a 24 hour period.

2.9.2. Hybridisation of radiolabelled probe to membrane

Membranes were prehybridised at 42°C for 30 minutes in preheated (68°C) ULTRAhyb™ buffer (Ambion). Radiolabelled probe was added to the hybridisation mix at a final concentration of 0.5 - $1x10^6$ cpm/ml buffer. Hybrisation was allowed to continue for overnight (14-24 hours) at 42°C.

2.9.3. Post-hybridisation washes

ULTRAhyb™ buffer was discarded and membranes were washed twice for 10 minutes at 42°C with buffer containing 2x SSC and 0.1% w/v SDS and then twice for 20 minutes at 50°C in buffer containing 0.1xSSC and 0.1% w/v SDS. For more stringent washes the second wash buffer (0.1xSSC and 0.1% w/v SDS) was repeated for 2x20 minutes at 60°C.

2.9.4. Development of signal

After washing, membranes were wrapped in clingfilm. The membrane was then exposed to X-ray film (XAR-5 or X-Omat S; Kodak) in cassettes with Du Pont enhancing screens at -70°C. After a specific exposure time the signal was developed using LX 24 developer and fixed using FX 40 fixative according to the suppliers recommendations (both Kodak). In order to quantify signals, membranes were placed in stormscanner cassettes containing storage phosphor screens (Molecular Dynamics, Sunnydale, CA, U.S.A) for specific exposure times. The relative optical density of the hybridised signals (both specific probes and 18S standards) were determined on a Storm™ optical scanner (Molecular Dynamics) using ImageQuant™ program (Molecular Dynamics). The 18S standard was used to correct for loading variations in the RNA samples.

2.10. Immunohistochemistry

2.10.1. Tissue pretreatment and primary antibody

Paraffin tissue sections prepared as described in section 3.4. and mounted onto glass slides. Sections were dewaxed in histoclear for approximately 5 minutes and rehydrated in decreasing concentrations of alcohol (100%, 96% and 70% respectively). The tissue sections were placed in 3% hydrogen peroxide in methanol for 30 minutes to reduce endogenous peroxidase activity. Slides were then washed in 0.05M Tris-HCl buffer pH7.4 containing 0.85% w/v NaCl (TBS) for 5 minutes. If antigen retrieval (details given in appropriate chapters), was required for the detection of the protein by the primary antibody it was carried out at this point. The tissue sections were then incubated with normal serum from the species in which the secondary antibody was raised (usually swine or rabbit) and diluted 1:5 in TBS for 30 minutes at room temperature. After this incubation, the normal serum was removed carefully from the sections with a tissue and replaced with primary antibody (in 1:5 normal serum: TBS) at an appropriate dilution, details of which are given in relevant chapters. Tissue was incubated with the primary antibody overnight at 4°C in a humidified chamber. The following day excess antibody was removed by two 5 minute washes with TBS.

2.10.2. Secondary antibody and detection system

The secondary antibodies for the HRP detection system were biotinylated for subsequent conjugation to avidin-linked peroxidase enzyme complexes. Secondary antibodies were diluted 1:500 in TBS and incubated on the tissue sections for 30 minutes. Excess antibody was removed by 2x5 minute washes with TBS. Sections were incubated in the presence of avidin-biotin horseradish peroxidase complex (Dako, Glostrup, Denmark) for 30 minutes. This bound to the biotinylated secondary antibody and formed a complex which served to amplify the signal obtained from the primary antibody. The solution was prepared according to the suppliers instructions in 0.05M Tris-HCl pH7.4 at least 20 minutes before use. Excess complex was removed by 2x5 minute washes in TBS and bound antibody visualised with a solution of 225μM DAB in 0.05M Tris/HCl, pH7.6 containing 0.01% hydrogen peroxide. Sections were washed in tap water and counterstained in haemotoxylin. They were subsequently dehydrated, cleared in xylene and mounted in pertex mounting fluid (Solmedia Lab Suppliers, Essex, U.K.) with coverslips.

Chapter 3.

Expression of Maturational Markers During Development of the Postnatal Rat Testis.

3.1. Introduction

During postnatal development in the rat, testicular cell populations undergo a number of transformations critical for the establishment of efficient spermatogenesis and the production of adequate amounts of testosterone by the adult testis. The fetal Leydig cell population declines in number and function after birth (Mendis-Handagama *et al.*, 1987). The Sertoli cell population ceases proliferation in parallel with proliferation and differentiation of adult Leydig cells (Clermont and Perey, 1957; Hardy *et al.*, 1989; Saez, 1994; Steinberger and Steinberger, 1971; Zhengwei *et al.*, 1990). The first wave of spermatogenesis takes place with an increase in the number of spermatogonia, the spermatocyte population becomes active followed by an expansion in spermatid numbers (Clermont and Perey, 1957). A significant amount of apoptosis and cell loss is also associated with the onset of spermatogenesis.

Postnatal development of the testis is a tightly regulated process controlled by endocrine factors and paracrine and autocrine interactions. The functional maturation of the Sertoli cell plays a pivotal role within this process. However Sertoli cell differentiation cannot begin until the cell has ceased dividing. In neonatal hypothyroidism Sertoli cell differentiation is delayed due to a prolonged period of cell replication and this results in a delay in the onset of germ cell proliferation and lumen formation (van Haaster *et al.*, 1992). Proteins secreted by immature Sertoli cells inhibit differentiation of Leydig cell precursors and consequently mature Sertoli cells affect/modify Leydig cell development via the production of paracrine factors (Kerr and Sharpe, 1985; Perrard-Sapori *et al.*, 1987).

The control of postnatal testicular development is a complicated process and is not yet fully understood. Numerous changes in key interactions involving Sertoli cells arise during this time; the hypothalamic-pituitary-testicular axis is established and a number of intratesticular paracrine interactions are set up in addition to altered endocrine environments and changing spermatogenic cell complements. In order to further investigate the regulation of postnatal testicular development additional markers of testicular functional maturation need to be established. Thus the aims of this study were to investigate normal development of the postnatal rat testis with emphasis on maturational changes in Sertoli cell protein expression. The expression patterns of known and potential markers of Sertoli cell functional maturation were studied at the level of gene transcription and protein expression. It was hoped that in future studies these preliminary results could be used to better define the stages of postnatal testicular development. Detailed markers of Sertoli cell development and maturation are essential in studies in which testicular development has been modified by exogenous agents (Sharpe *et al.*, 1998). Sertoli cell proliferation within the rat testis was also studied in order to establish when Sertoli cell mitosis ceased and subsequent cell maturation began in the testes of the rats being studied in this report. Examination of spatial and temporal changes in oestrogen and androgen receptors was also carried out to determine the sites of direct steroid action in the postnatal testis as it was hoped that in future studies the effect of oestrogen and testosterone on Sertoli function would be investigated.

Five proteins expressed in postnatal Sertoli cells were studied:

SGP-1. SGP-1 is one of the most abundant Sertoli cell products detectable by gel electrophoresis. mRNA levels peak between postnatal days 10 and 20 after which levels decline such that in the adult levels of SGP-1 mRNA are similar to those in day 3 Sertoli cells (Mathur *et al.*, 1994). SGP-1 expression by Sertoli cells does not appear to be regulated by hormones or germ cell complement (Mathur *et al.*, 1994). It has been proposed that the pattern of SGP-1 expression in Sertoli cells is dependent upon the chronological age of the Sertoli cell rather

than the maturational stage of the cell (Bunick *et al.*, 1994). SGP-1 was therefore included in these studies as a possible marker of Sertoli cell 'age' rather than as a marker of Sertoli differentiation.

Inhibin-alpha (α). The alpha subunit of both inhibins A and B is produced by Sertoli cells and both mRNA and protein expression of inhibin-α is greatest during immature testis development (Gondos and Berndston, 1994). Inhibin-α levels have been extensively used as a marker of Sertoli cell function and development due to the fact that expression becomes stage specific as the Sertoli cell matures and germ cell complement increases (Majdic *et al.*, 1997). Inhibin-α was included in the present study as a well described and researched marker of Sertoli cell functional maturation and could therefore be used as a standard by which to compare other less well known Sertoli cell proteins.

Musashi-1. The RNA binding protein, Musashi-1 is expressed in Sertoli cells of fetal, immature and adult testis. Expression is both cytoplasmic and nuclear. However with the onset of Sertoli cell maturation preliminary studies have suggested that Musashi-1 expression becomes predominantly nuclear (Maguire *et al.*, 1999). In the adult testis Musashi-1 expression is most abundant in tubules at spermatogenic stages where expression levels of androgen receptor were lowest (stages XI-VI) (Bremner *et al.*, 1994; Maguire *et al.*, 1999). For these reasons Musashi-1 was investigated as a novel marker of Sertoli cell functional maturation with the possibility of steroid or germ cell regulated expression.

GATA-1. Testicular expression of the zinc finger transcription factor GATA-1 is restricted to Sertoli cell nuclei (Ketola *et al.*, 1999; Yomogida *et al.*, 1994) and reaches maximal levels of expression between 14 and 23 days of age (Viger *et al.*, 1998). GATA-1 expression becomes stage dependent with the onset of the first wave of spermatogenesis. Expression of GATA-1 in the adult testis is restricted to stages VII-IX (Yomogida *et al.*, 1994). These authors suggested that expression may be inhibited by certain germ cell populations and/or upregulated by testosterone. GATA-1 was included in this study as a possible

marker of Sertoli cell maturation as expression is upregulated when Sertoli cell differentiation begins and expression is possibly regulated by germ cells and/or steroids. GATA-1 has also been shown to upregulate inhibin-α expression in Leydig cells (Feng *et al.*, 1998) and could therefore play an important role within the differentiated Sertoli cell.

GATA-4. GATA-4 is another member of the GATA zinc finger family of transcription factors and is expressed in Sertoli and Leydig cells of fetal and immature testes. GATA-4 Sertoli cell mRNA levels are maximal up to postnatal day 14 after which levels begin to decline alongside increasing Sertoli cell maturation (Ketola *et al.*, 1999). There is some disagreement regarding GATA-4 expression in adult testis: Heikinheimo et al., (1997) and Ketola et al., (1999) state that although mRNA levels are low in the adult testis, GATA-4 continues to be expressed in both Sertoli and Leydig cells. Viger et al., (1998) disagree and state that as Sertoli cell proliferation ceases GATA-4 expression switches from the Sertoli cell to the germ cells. It has also been suggested that GATA-1 'takes over' the function of GATA-4 within the Sertoli cell at this point (Viger *et al.*, 1998). During this study it was hoped to determine if GATA-4 continued to be expressed in Sertoli cells following the onset of maturation or if the transcription factor was a marker of immature proliferating Sertoli cells where expression declined with increasing differentiation.

Expression patterns of steroid receptors in the postnatal rat testis were also studied;

Androgen receptor (AR)

Levels of AR protein and mRNA have been previously shown to increase as Sertoli cells develop and expression becomes stage dependent in the adult Sertoli cell (Bremner *et al.*, 1994; Majdic *et al.*, 1995). Therefore AR expression in Sertoli cells can be used as a marker of cell maturation.

Oestrogen receptor alpha (ERα)

It is generally agreed that ERα expression in the postnatal rat testis is restricted to fetal and adult type Leydig cells (Fisher *et al.*, 1997).

Oestrogen receptor beta (ERβ)

ERβ expression had not been investigated prior to this study but ERβ expression has since been observed in Sertoli cell nuclei throughout postnatal testis development in the rat (Saunders *et al.*, 1998; van Pelt *et al.*, 1999).

3.2. Experimental Procedures

3.2.1. Preparation of cDNA probes

3.2.1.1. SGP-1

A 353bp cDNA for rat SGP-1 contained in a pCRII-TOPO vector had been previously constructed 'in house' (Millar *et al.*, 1995). The cloned SGP-1 cDNA insert was prepared for radiolabelling as described in section 2.8.1.

3.2.1.2. Inhibin-α

A 432bp cDNA for rat inhibin-α had been previously cloned 'in-house' into Bluescript vector (Pineau *et al.*, 1990). The cDNA insert was prepared for radiolabelling as described in section 2.8.1.

3.2.1.3. Musashi-1

A 413bp cDNA for rat Musashi-1 was obtained from Dr Sharon Maguire who had amplified the partial cDNA from rat ovarian cDNA using sequence specific primers. The cDNA was contained in pCRII-TOPO vector and was prepared for radiolabelling as described in section 2.8.1.

3.2.1.4. GATA-4

Oligonucleotide primers were selected by Dr Philippa Saunders to amplify from base 1198 to base 1860 of the published sequence of mouse GATA-4 (Arceci *et*

al., 1993). The oligonucleotide sequences were; 5'primer 5'TTG ACG ACT TCT CAG AAG GCA G3' (bases 1198-1219) and 3' primer 5'CCG ACA AGG TTC TCA GGA C3' (bases 1842-1860). The cDNA was amplified by PCR from a pool of day 10 rat testis cDNAs prepared by random priming (section 2.5.2). Using the PCR mix as in section 2.5.2 thirty five amplification cycles were performed with an annealing temperature of 60°C for 35 seconds and a 45 second extension at 72°C. Following amplification the 663bp partial cDNA was purified, sequenced and subcloned into pCRII-TOPO vector (see Chapter 2). The rat GATA-4 partial cDNA had 96.2% sequence homology with the published mouse GATA-4 cDNA over this region of sequence. The cloned cDNA was prepared for radiolabelling as described in section 2.8.1.

3.2.1.5. GATA-1

Oligonucleotide primers were selected by Dr Philippa Saunders to amplify from base 2417 to base 3045 of the published sequence of rat GATA-1 cDNA (Ito *et al.*, 1993). The oligonucleotide sequences were; 5'primer 5'GTT TGT GGA TTC TGC CCT GGT GTC3' (bases 2417-2440) and 3' primer 5'CAT TGC ACA GGT AGT GAC CTG TCC3' (bases 3022-3045). The cDNA was amplified by PCR from a pool of day 21 rat testis cDNAs prepared by reverse transcription using oligo dT primers (section 2.5.2). Using the PCR mix as in section 2.5.2 thirty five amplification cycles were performed with an annealing temperature of 60°C for 35 seconds and a 45 second extension at 72°C. Following amplification the 627bp partial cDNA was purified, sequenced and subcloned into pCRII-TOPO vector (see Chapter 2). The cloned cDNA was prepared for radiolabelling as described in section 2.8.1.

3.2.1.6. 18S ribosomal RNA

A 423bp cDNA for rat 18S which had been previously cloned into a pCRII-TOPO vector was obtained from Dr. Katie Turner. The cDNA was prepared for radiolabelling as described in section 2.8.1.

3.2.2. RNA extraction and separation

Total RNA was extracted from rat tissue and separated on a 1.5% denaturing agarose gel as described in sections 2.3.2. and 2.3.3., respectively. RNA was obtained from testis of rats aged between postnatal day 1 to adult rats (which were older than postnatal day 70). For each age group used, testes were obtained from two separate litters. Due to the small size of testes from rats aged postnatal days 1 and 5, RNA from these litters was pooled.

3.2.3. Northern blot analysis

Separated total RNA was transferred onto a nylon membrane as described in section 2.9.1. and Northern blot analysis was performed as in section 2.9.

3.2.4. Bromodeoxyuridine (BrDU) Treatment

BrDU (Sigma) treatment of immature Wistar rats was very kindly carried out by Dr. Richard Sharpe as described in Sharpe et al., (1999). Briefly, groups of four to six male Wistar rats aged postnatal day 3 to day 21 were administered 100mg/kg of BrDU 1 hour before the rats were killed as described in section 2.2.1. BrDU is a thymidine analogue and was used to label cells undergoing DNA replication during the S phase of the cell cycle.

3.2.5. Tissue Collection and Fixation

Rats testes were collected and fixed as described in Chapter 2. At the mid point of fixation testes were weighed and testis weight was recorded in mg. Tissue sections were cut and prepared for immunocytochemical staining as described in sections 2.4. Testes fixed for immunocytochemical staining were from a minimum of two different animals per age group and were analysed on the same slide.

3.2.6. Immunocytochemical staining

Immunocytochemical staining was performed as described in section 2.10. Specific details for each antibody are given below:

3.2.6.1. Markers of cell division

Three different antibodies were used to study cell proliferation during prepubertal development;

Proliferating cell nuclear antigen (PCNA). PCNA functions as an auxiliary protein to DNA polymerase delta (Bravo *et al.*, 1987) and is strongly expressed in cells during G1 and S phases of the cell cycle. Weaker PCNA expression also occurs during the M phase (Kurki *et al.*, 1986). A mouse monoclonal antibody raised against PCNA (DAKO) was used at a dilution of 1:100 and biotinylated rabbit anti-mouse immunoglobulins were used as secondary antibodies. No antigen retrieval was carried out.

Ki67. Ki67 is abundantly expressed during the G2 and M phases of the cell cycle. A monoclonal antibody raised against Ki67 in the mouse (Novocastra) was used at a dilution of 1:50 and biotinylated rabbit anti-mouse immunoglobulins were employed as the secondary antibody. Tissue sections were subjected to antigen retrieval prior to the application of primary antibody. Slides were pressure cooked for 5 minutes in pre boiled 0.01M citrate buffer pH 6. The pressure was then released and sections were allowed to stand for 20 minutes undisturbed before washing twice in TBS for 5 minutes.

BrDU. An anti-BrDU mouse monoclonal antibody (Boehringer) was used at a dilution of 1:30. Biotinylated rabbit anti-mouse immunoglobulins were applied as the secondary antibody. Antigen retrieval on tissue sections was carried out as for Ki67.

3.2.6.2. Markers of testicular development

SGP-1. A rabbit polyclonal antibody, kindly donated by Dr. Steven Sylvester (University of Washington, Seattle) was used to immunolocalise SGP-1 to tissue sections. The antibody was used at a dilution of 1:1000 and biotinylated swine anti-rabbit immunoglobulins were used as the secondary antibody. No antigen retrieval was carried out.

Inhibin-α. Immunolocalisation of inhibin–α was carried out using a monoclonal antibody (173/9K) which was a gift from Dr. Nigel Groome (Oxford Brookes University, Oxford). The antibody was used at a dilution of 1:450 and the secondary antibody was biotinylated rabbit anti-mouse immunoglobulin. Antigen retrieval was carried out as for Ki67.

Musashi-1. Dr. Sharon Maguire kindly carried out the immunolocalisation of Musashi-1 to rat testes tissue sections using an anti-mouse–Musashi-1 monoclonal antibody raised in the rat according to standard procedures using a GST-mouse-Musashi-1 fusion protein (Sakakibara *et al.*, 1996). The antibody was a gift from Professor Okano (Osaka University, Japan) to Dr. Philippa Saunders and was used at a dilution 1:25000. Biotinylated rabbit anti-rat immunoglobulins (Vector, Peterborough, U.K.) were used as the secondary antibody. Tissue sections were subjected to antigen retrieval as given for Ki67. An avidin-biotin block using the Avidin/Biotin blocking kit from Vector was carried out to reduce non-specific interstitial staining. 4 drops of the Avidin D blocking solution was added per ml of normal rabbit blocking serum which was left on the tissue sections for 30 minutes followed by 2 x 5 minute TBS washes. 4 drops of Biotin solution per ml of TBS used was added to the slides for 15 minutes followed by two additional 5 minute TBS washes. The primary antibody was then added and immunocytochemical staining was continued as in section 2.10.

GATA-4. A goat polyclonal anti-rat GATA-4 antibody (SantaCruz) was used at a dilution of 1:500. Biotinylated rabbit anti-goat immunoglobulins (DAKO) were utilised as secondary antibodies. Antigen retrieval was carried out as described for Ki67.

GATA-1. A rat monoclonal antibody against GATA-1 (Santa Cruz) was used at a dilution of 1:500 and biotinylated rabbit anti-rat immunoglobulins (Vector) were used as the secondary antibody. Tissue sections were subjected to antigen retrieval as described for Ki67. Due to non-specific interstitial staining an

Avidin/Biotin block was carried out between antigen retrieval and primary antibody addition, as described for Musashi-1.

3.2.6.3. Steroid hormone receptors

Androgen receptor (AR). A rabbit polyclonal antibody (Santa Cruz) was used to immunolocalise AR to the tissue sections. The antibody was used at a dilution of 1:200 and antigen retrieval was carried out as for Ki67. In place of the biotinylated secondary antibody and avidin-conjugated horseradish peroxidase complex, DAKO EnVision + System (anti rabbit) with peroxidase (DAKO) was applied to the sections for 30 minutes, following manufacturers instructions. The system is based on an HRP labelled polymer which is conjugated to an anti-rabbit secondary antibody resulting in a more sensitive detection system. DAB was then applied to the sections as in section 2.10.2.

Oestrogen receptor beta (ERβ). A sheep polyclonal antibody raised against a peptide within the hinge domain of human ERβ was generated in house by Dr. Philippa Saunders (Saunders *et al.*, 2000). The ERβ antibody was used at a dilution of 1:1000. Biotinylated rabbit anti-sheep immunoglobulins were employed as secondary antibody (Vector). Antigen retrieval was carried out as for Ki67 except that 0.01M glycine pH 3.5 was used in place of 0.01M citrate buffer pH6.

Oestrogen receptor alpha (ERα). A monoclonal anti-human ERα antibody (Novacastra) was utilised to immunolocalise ERα. The antibody was used at a dilution of 1:20 and antigen retrieval was carried out as for Ki67. DAKO EnVision + System (anti mouse) with peroxidase (DAKO) was used in place of secondary antibody and conjugated horseradish peroxidase. The system is based on an HRP labelled polymer which is conjugated to an anti-mouse secondary antibody resulting in a more sensitive detection system. DAB was then applied to the sections as in section 2.10.2.

The specificity of the specific antibodies (except for ERβ) was checked by replacing each antibody with normal serum from the animal species that the primary antibody was raised in. The normal serum was diluted to ensure protein content was the same as in the diluted primary antibody. In the case of ERβ, the peptide to which the primary antibody was raised was incubated in the presence of the primary antibody overnight at 4°C. The concentration of incubated peptide was ten times that of the antibody with regards to protein content. The pre-absorbed antibody was then diluted to the working concentration which corresponded to that of the primary antibody. None of the above controls resulted in any signal above background.

Sections were analysed using an Olympus Provis microscope and images were captured onto computer using either a Kodak DCS420 camera or a Kodak DCS330 camera and images were stored on the computer using the Photoshop 5.0 programme (Adobe).

3.3. Results

3.3.1. Sertoli cell multiplication

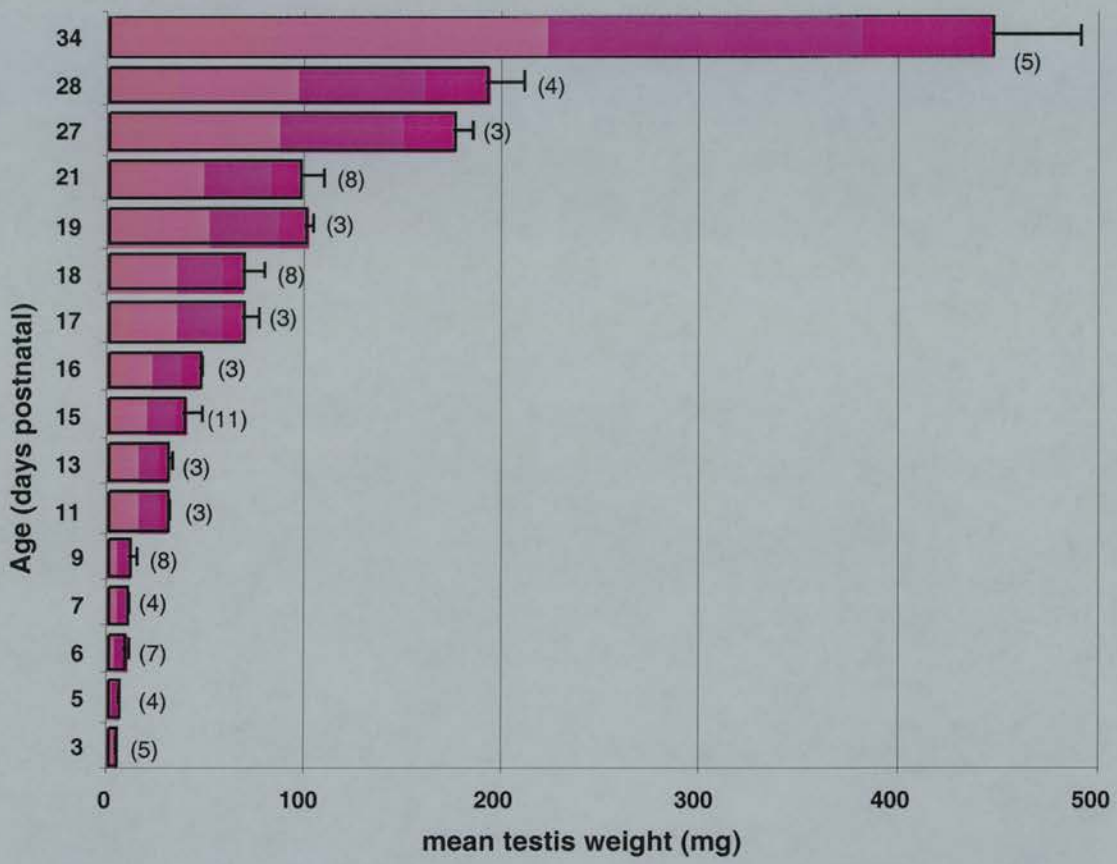

Figure 3.1 Changes in mean testis weight during postnatal development. Graph to show changes in weight of testis during postnatal development in the rat. Testes removed from rats aged between postnatal day 3 and 34 were weighed (mg). Mean testes weight for each age group was calculated and the number of animals per age group are shown in brackets.

Testes removed from rats aged postnatal day 3 to day 34 were weighed and mean testis weight in mg for each age were calculated and are presented in Figure 3.1. The bar chart clearly shows that testis weight remained fairly constant between days 3 and 9 of postnatal life but thereafter more than doubled over the next 12 days such that by day 28 testes weighed nearly fifty

times that at day 3. By day 34, testis weight had further increased at least one hundred fold compared to testis weight at day 3.

3.3.1.1. PCNA

Immunocytochemical staining with anti-PCNA antibody resulted in a strong immunopositive signal restricted to the nuclei of cells. At all ages examined there were immunonegative cells within the interstitium. However there was strong immunolocalisation of PCNA to some interstitial cells in day 3 and day 9 testes (Fig. 3.2.a, b). Numbers of positively stained interstitial cells appeared to be reduced in day 15 and 16 testes (Fig. 3.2.c, d) but increased again in day 18 and 21 testis (Fig. 3.2.e, f). In day 3 testes the majority of cells in the seminiferous tubules were strongly immunopositive for PCNA (Fig. 3.2.a). The number of immunopositive Sertoli cells was reduced as postnatal development progressed such that by day 15 and 16 the majority of Sertoli cell nuclei were immunonegative for PCNA (Fig. 3.2.c, d, arrows). The number of immunopositive Sertoli cells continued to decline although at day 21 a small number of Sertoli cells remained immunopositive (Fig. 3.2.f, black arrowheads). In the adult testes all Sertoli cell nuclei were immunonegative for PCNA (Fig. 3.2.g). Immunopositive spermatogonia were first observed in day 9 testes (Fig. 3.2.b blue arrowheads). Thereafter increasing numbers of immunopositive spermatogonia were observed as germ cell numbers increased. With the first wave of spermatogenesis some immunopositive primary spermatocytes could be observed in day 18 rat testes. The number of positively stained spermatocytes was increased by day 21 (Fig. 3.2.f). However in the adult testis spermatogonia were the only immunopositively labelled cells (Fig. 3.2.g, blue arrowheads).

3.3.1.2. Ki67

An intense immunopositive signal for Ki67 antibody was restricted to the nuclei of dividing cells. A large proportion of interstitial cells were stained immunopositively for Ki67 in day 3 testes (Fig. 3.3.a). By day 18 the number of positively stained interstitial cells had declined (Fig. 3.3.c) and remained low in day 21 testes (Fig. 3.3.d). The majority of cells within the seminiferous tubules

of day 3 testes were strongly immunostained for Ki67 (Fig. 3.3.a, arrowheads). By day 14 the majority of Sertoli cells were immunonegative (Fig. 3.3.b, arrows) and in day 18 testes all Sertoli cell nuclei were immunonegative for Ki67 (Fig. 3.3.c, arrows). Spermatogonia had weak Ki67 immunostaining in day 3 testes (Fig. 3.3.a). However by day 14 (Fig. 3.3.b) the intensity of staining and the number of immunopositive spermatogonia had increased such that by day 18 and 21 the majority of spermatogonia were immunopositive (Fig. 3.3. c, d). Ki67 was first localised to primary spermatocytes in day 18 testes and immunostaining of primary spermatocytes was still present in day 21 testis (Fig. 3.3. c, d).

3.3.1.3. BrDU

The anti-BrDU antibody localised strongly to nuclei of cells in the S phase of mitosis. Non uniform BrDU labelling of interstitial cells was observed in day 3, day 5 and day 8 testes (Fig. 3.4.a-c). The number of labelled interstitial cells was low in day 10 testis. However some interstitial cells were immunopositive for BrDU in day 15 and day 21 testes (Fig. 3.4.e,f). Some Sertoli cells had strong BrDU labelling in day 3 testes. However the intensity of labelling was variable and some cells were immunonegative for BrDU at this age (Fig. 3.4.a). In day 5, day 8 and day 10 rat testes (Fig 3.4 b-d) there were numerous positively labelled Sertoli cell nuclei. However by day 15 (Fig 3.4.e) this number had markedly declined and the majority of Sertoli cells had no BrDU labelling; in day 21 testis no positively labelled Sertoli cell nuclei could be seen (Fig 3.4.f). In day 15 testes weak BrDU labelling of spermatogonia could be observed in some tubules. By day 21 BrDU labelling had become stronger and the number of labelled spermatogonia had increased. The first labelled primary spermatocytes were also seen at this age however some seminiferous tubules did not contain any positively labelled germ cell (Fig. 3.4. f, asterisks).

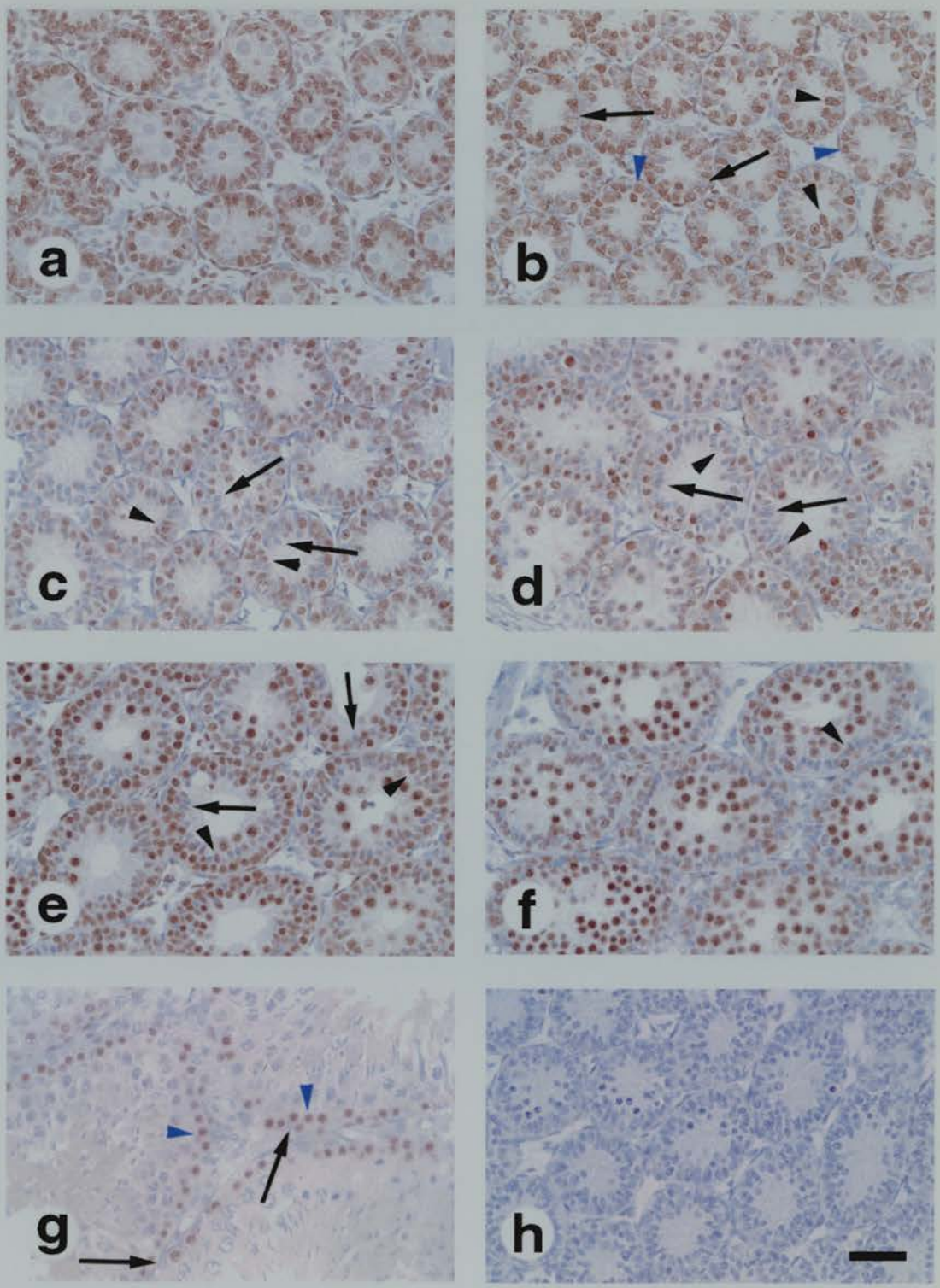

Figure 3.2: Immunolocalisation of PCNA to dividing cells in the postnatal rat testis. Testes obtained from rats aged postnatal day 3 **(a)**, day 9 **(b)**. day 15 **(c)**, day 16 **(d)**, day 18 **(e)**, day 21 **(f)**, and adult **(g)** are shown. A representative negative control using normal mouse IgGs in place of primary antibody on day 15 testis is also shown **(h)**. Arrowheads point to immunopositive Sertoli cell nuclei. Blue arrowheads point to immunopositive spermatogonia. Arrows point to immunonegative Sertoli cell nuclei. Scale bar represents 50μm.

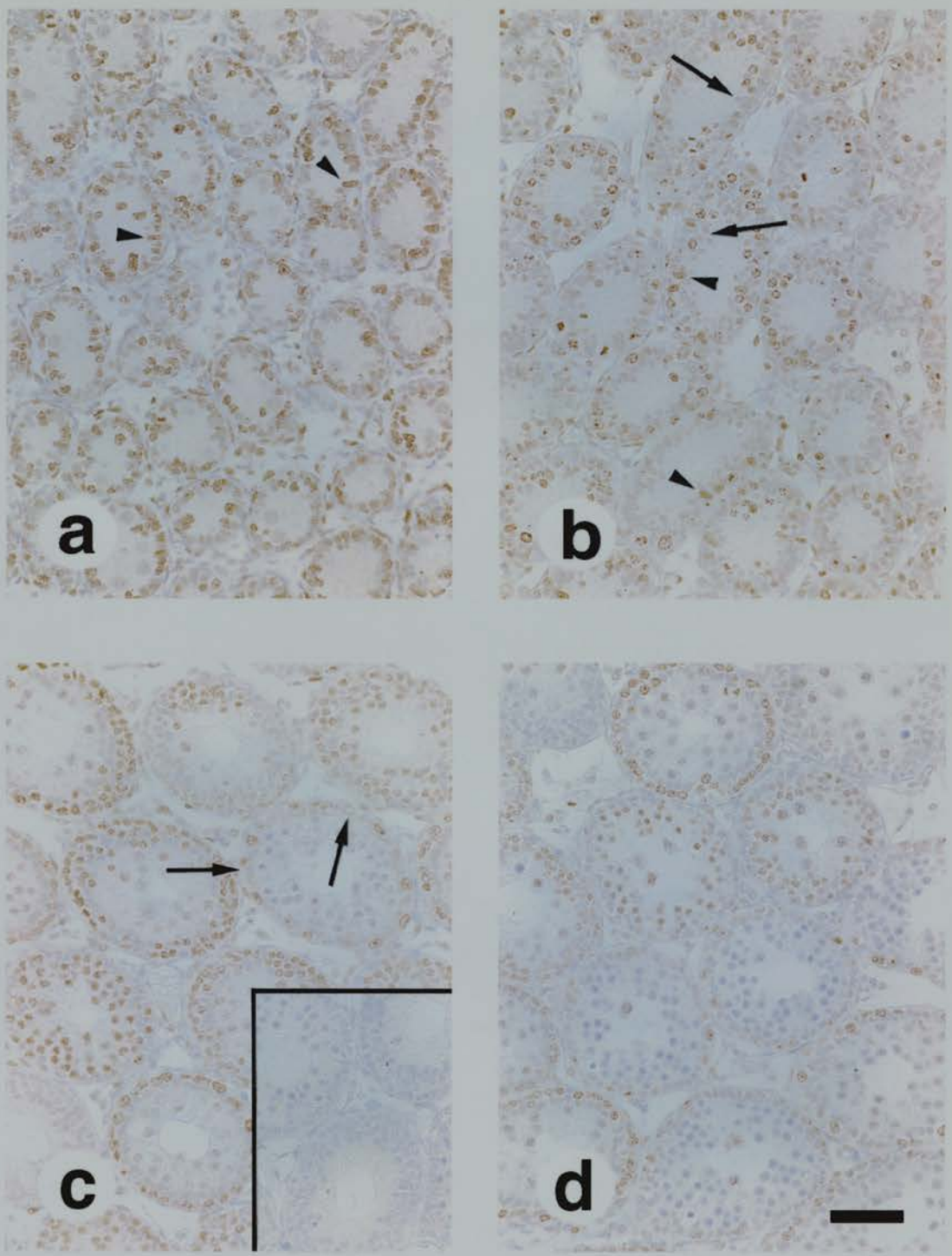

Figure 3.3: Immunolocalisation of Ki67 to dividing cells in the postnatal rat testis. Testes from rats aged postnatal day 3 **(a)**, day 14 **(b)**, day 18 **(c)** and day 21 **(d)** are shown. Inset shows representative negative control when primary antibody was replaced with normal mouse IgGs. Arrowheads point to immunopositive Sertoli cell nuclei, arrows point to Sertoli cell nuclei with low Ki67 immunostaining. Scale bar represents 50μm.

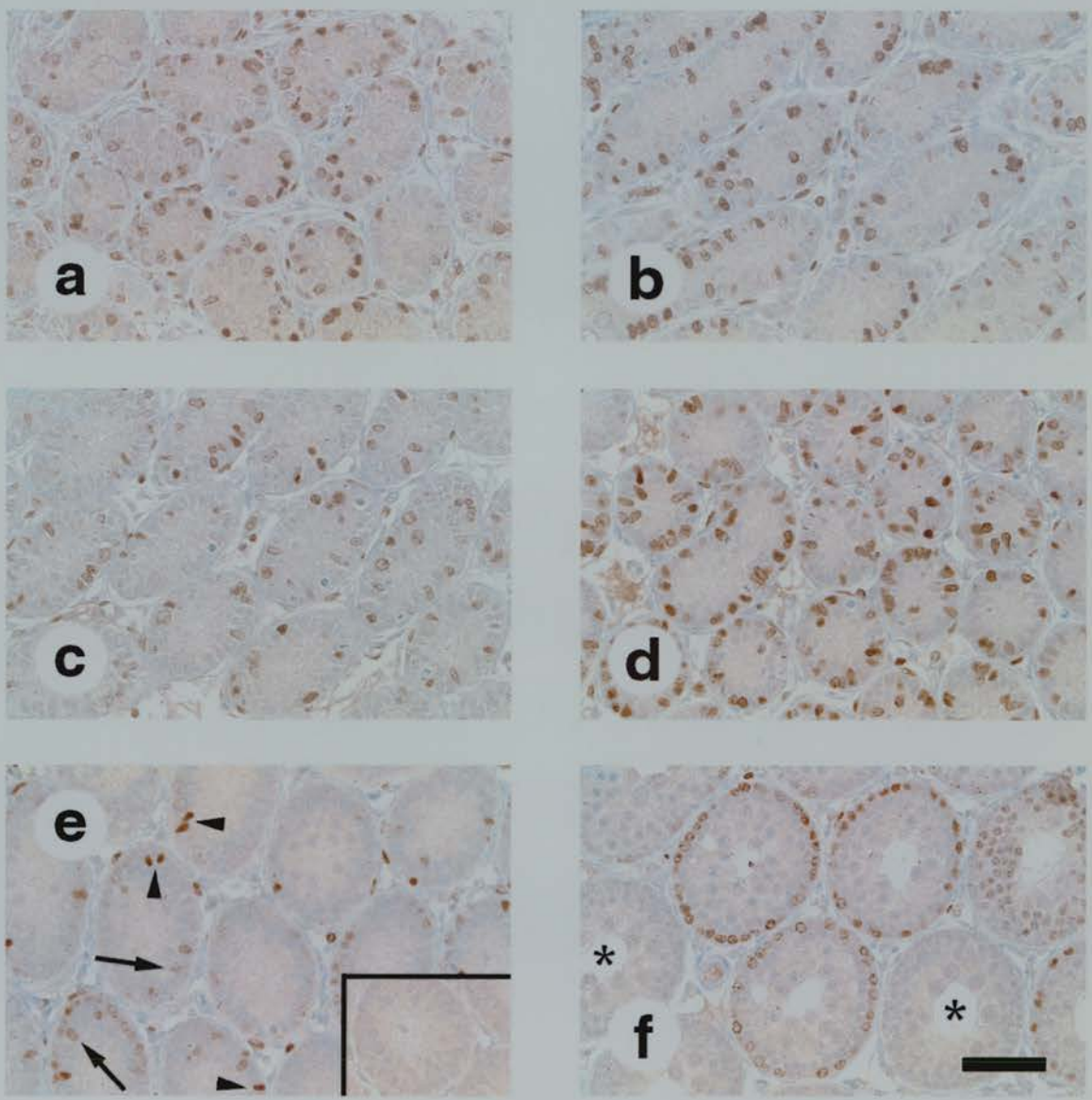

Figure 3.4: BrDU labelling of proliferating cells in the postnatal rat testis. Testes from rats aged postnatal day 3 **(a)**, day 5 **(b)**, day 8 **(c)**, day 10 **(d)**, day 15 **(e)** and day 21 **(f)** are shown. Inset shows a representative negative control when primary antibody was replaced with normal mouse IgGs. Arrowheads point to strongly labelled Sertoli cell nuclei, arrows point to Sertoli cell nuclei with low BrDU labelling. Asterisks mark tubules containing immunonegative cells only. Scale bar represents 50μm.

3.3.2. mRNA expression in the postnatal Sertoli cell

3.3.2.1. SGP-1

The cDNA probe specific for rat SGP-1 was found to hybridise to a 2.6kb transcript (as described in Collard et al., (1988) in testicular RNA from rats aged postnatal day 1 (birth) to day 70 (adult) (Fig 3.5A). The RNA was reprobed using a cDNA probe specific for rat 18S which hybridised to a 1.9kb transcript (Fig 3.5B). Signals for both transcripts were quantified (section 2.9.4.) and the SGP-1 signal was corrected for loading using the 18S signal (Fig 3.5 C). SGP-1 mRNA was rapidly upregulated after day 11 and maximal levels were reached between days 15 and 18. SGP-1 mRNA levels declined slightly after day 21 and by day 28 levels were similar to those seen in the adult testes.

3.3.2.2. Inhibin–α

Northern blot analysis using a cDNA probe for rat inhibin–α detected a 1.6kb transcript in testicular RNA from rats aged postnatal day 1 (birth) to day 70 (adult). A strong signal for the inhibin-α transcript was seen at all ages studied (Fig. 3.6A). Following 18S correction for loading error (Fig 3.6B) (section 2.9.4.) changes in inhibin-α mRNA expression in testis during postnatal development were examined (Fig. 3.6C). Inhibin-α mRNA levels slowly increased after birth up to day 15 after which there was a slight decline in levels at day 18. Inhibin-α mRNA expression was upregulated again in day 21 testes followed by a rapid decline in inhibin-α mRNA levels such that expression in the adult testis was a third of that at birth.

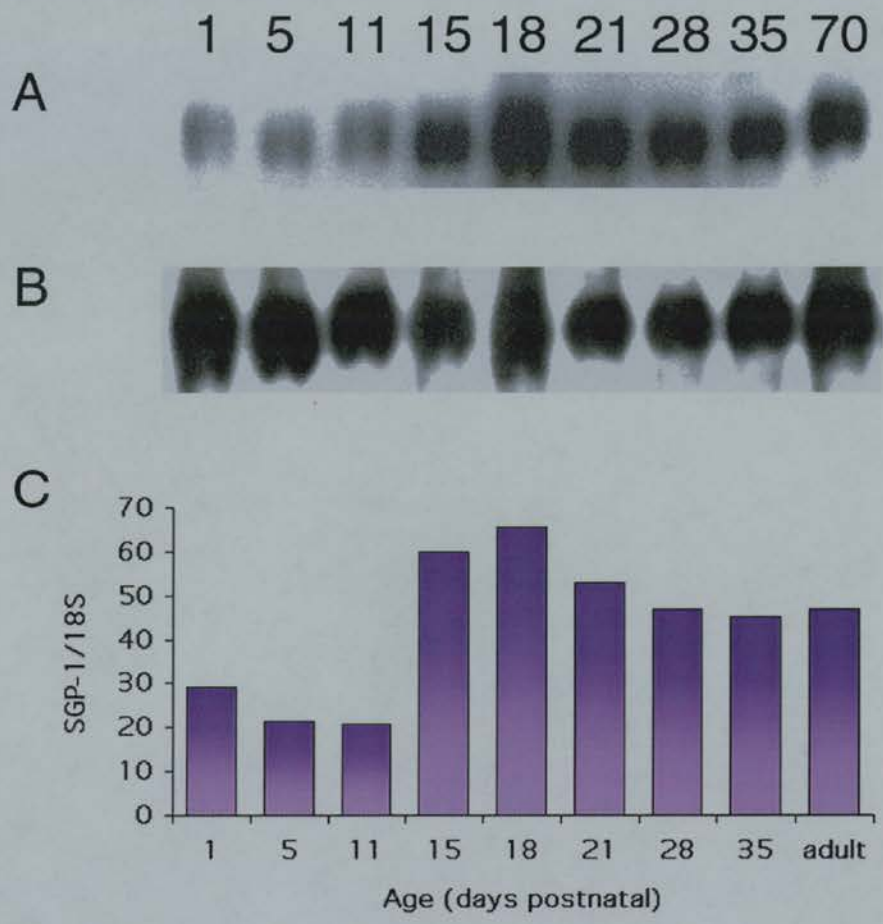

Figure 3.5: Northern blot analysis of SGP-1 mRNA expression during postnatal rat testis development. All lanes were loaded with 10 µg of total RNA extracted from testes obtained from rats aged postnatal day 1, 5, 11, 15, 18, 21, 28, 35 and adult (rats aged day 70 and over). RNA was hybridised with ^{32}P-labelled SGP-1cDNA which detected a 2.6kb transcript **(A)**. The RNA was reprobed with a ^{32}P-labelled 18S cDNA which hybridised to a 1.9kb 18S rRNA transcript and was used to check for even loading of RNA **(B)**. Following quantification of hybridisation signals the ratios of SGP-1 mRNA expression to 18S rRNA were plotted for each age to show changes in testicular SGP-1 mRNA expression during postnatal development **(C)**.

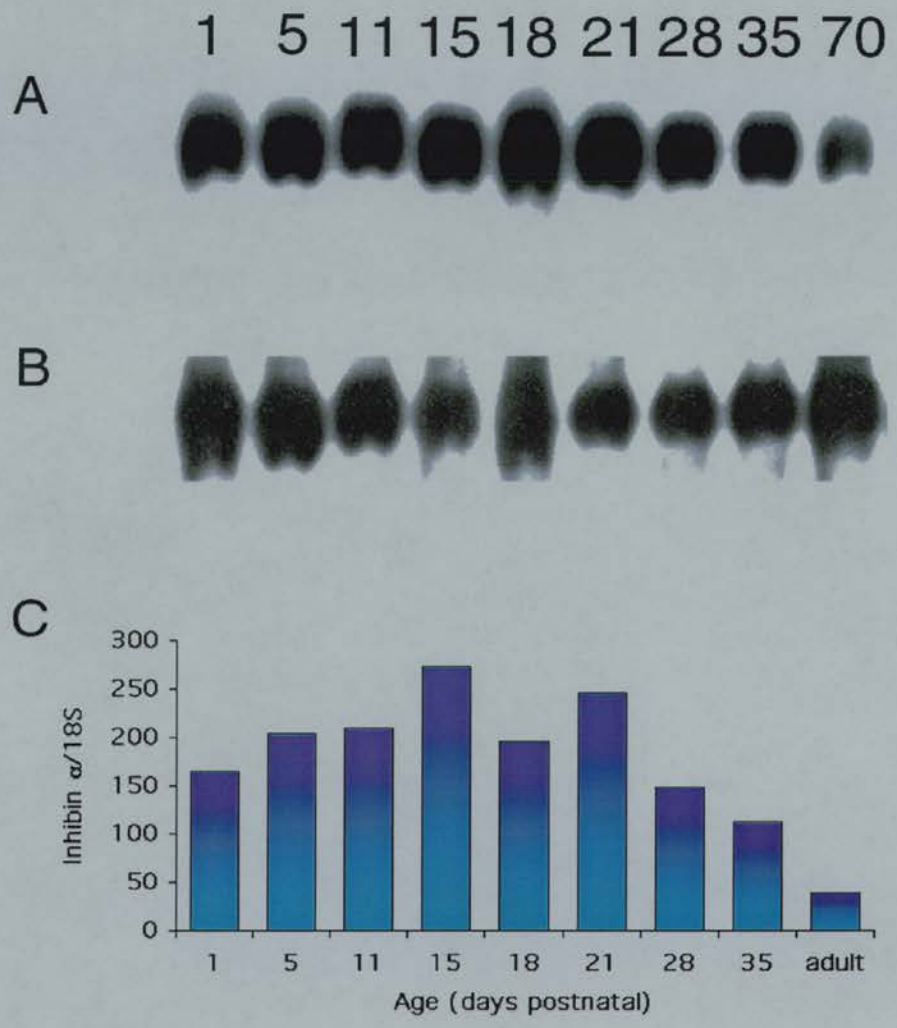

Figure 3.6: Northern blot analysis of inhibin-α mRNA expression during postnatal rat testis development. All lanes were loaded with 10 µg of total RNA extracted from rats aged postnatal day 1, 5, 11, 15, 18, 21, 28, 35 and adult (rats aged day 70 and over). RNA was hybridised with ^{32}P-labelled inhibin-α cDNA probe which detected a 1.5kb transcript **(A)**. The RNA was reprobed with a ^{32}P-labelled 18S cDNA which hybridised to a 1.9kb 18S rRNA transcript and was used to check for even loading of RNA **(B)**. Following quantification of hybridisation signals the ratios of inhibin-α mRNA expression to 18S rRNA were plotted for each age to show changes in testicular inhibin-α mRNA expression during postnatal development **(C)**.

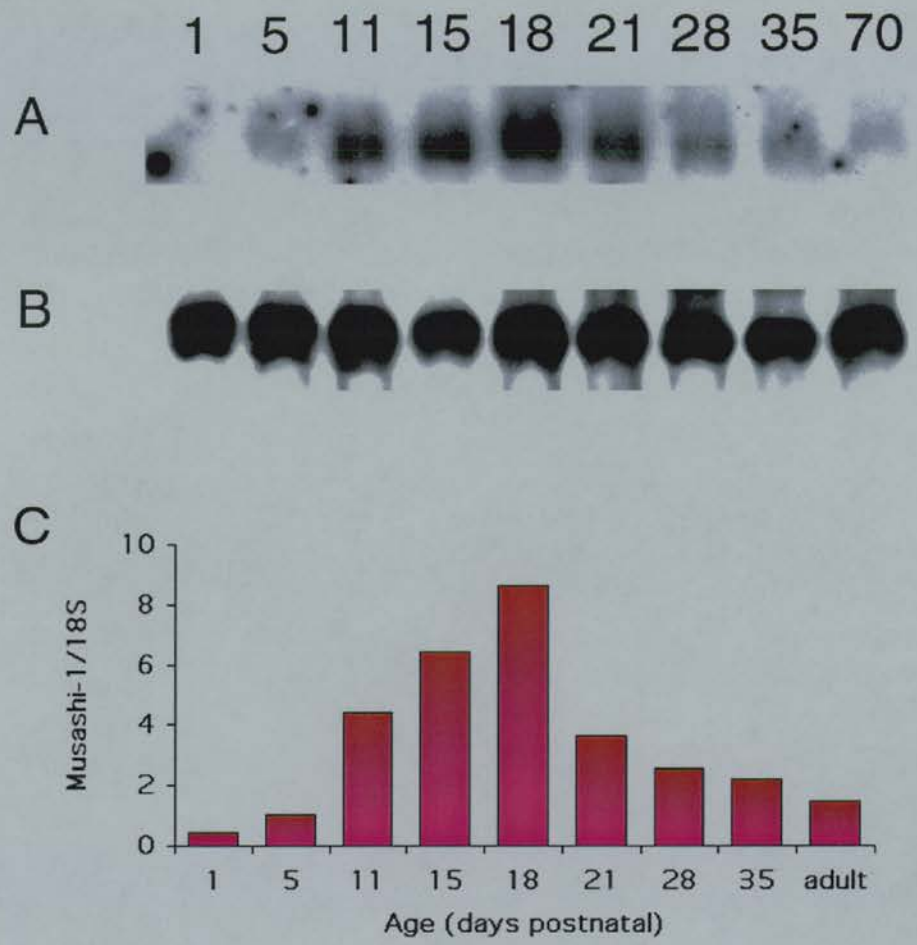

Figure 3.7: Northern blot analysis of Musashi-1 mRNA expression during postnatal rat testis development. All lanes were loaded with 10 µg of total RNA extracted from testes obtained from rats aged postnatal day 1, 5, 11, 15, 18, 21, 28, 35 and adult (rats aged day 70 and over). RNA was hybridised with ^{32}P-labelled Musashi-1 cDNA which detected a 3kb transcript **(A)**. The RNA was reprobed with a ^{32}P-labelled 18S cDNA which hybridised to a 1.9kb 18S rRNA transcript and was used to check for even loading of RNA **(B)**. Following quantification of hybridisation signals the ratios of Musashi-1 mRNA expression to 18S rRNA were plotted for each age to show changes in testicular Musashi-1 mRNA expression during postnatal development **(C)**.

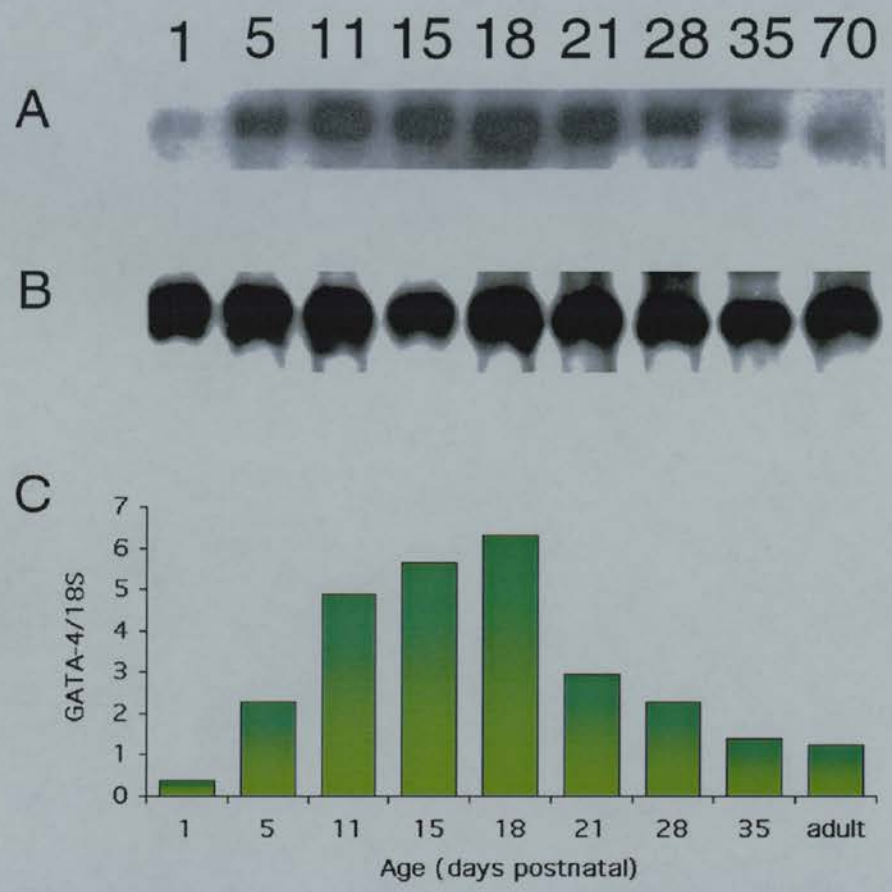

Figure 3.8: Northern blot analysis of GATA-4 mRNA expression during postnatal rat testis development. All lanes were loaded with 10μg of total RNA extracted from testes obtained from rats aged postnatal day 1, 5, 11, 15, 18, 21, 28, 35 and adult (rats aged day 70 and over). RNA was hybridised with ^{32}P-labelled GATA-4 cDNA which detected a 3.1kb transcript **(A)**. The RNA was reprobed with a ^{32}P-labelled 18S cDNA which hybridised to a 1.9kb 18S rRNA transcript and was used to check for even loading of RNA **(B)**. Following quantification of hybridisation signals the ratios of GATA-4 mRNA expression to 18S rRNA were plotted for each age to show changes in testicular GATA-4 mRNA expression durng postnatal development **(C)**.

3.3.2.3. Musashi-1

Using a cDNA probe specific for rat Musashi-1, a 3kb transcript was detected in testicular RNA from rats aged postnatal day 1 (birth) to day 70 (adult) (Fig. 3.7A). A cDNA probe for rat 18S was used to reprobe the RNA and a 1.9kb transcript was detected (Fig. 3.7B) and used to correct the quantified Musashi-1 hybridisation signal for loading error (Fig 3.7C). Between birth and day 18 there was an upregulation in Musashi-1 mRNA levels reaching maximal levels at day 18. Expression of Musashi-1 then rapidly declined and levels remained low into adulthood.

3.3.2.4. GATA-4

A single 3.1kb transcript for rat GATA-4 was detected in testicular RNA from rats aged postnatal day 1 to day 70 (Fig 3.8A). The signal for the GATA-4 transcript was quantified and corrected for 18S loading using the signal in Figure 3.8B. GATA-4 mRNA expression was rapidly upregulated after birth (Fig. 3.8C) and reached maximal levels by day 18. GATA-4 mRNA expression levels then declined and remained low into adulthood.

3.3.3. Immunoexpression of markers of testicular development

3.3.3.1. SGP-1

Immunolocalisation of SGP-1 showed cytoplasmic staining in a number of different testicular cell populations. In fetal day 20.5 and postnatal day 3 (Fig. 3.9. a, b) SGP-1 immunolocalisation was observed in cytoplasm of interstitial cells and the intensity of staining was non uniform between what appeared to be different cell populations. Interstitial immunolocalisation of SGP-1 remained strong up to day 15 (Fig. 3.9. d); thereafter the number of immunopositive interstitial cells declined such that in the adult testes all interstitial cells were immunonegative for SGP-1 (Fig. 3.9. g). Positive immunoreaction of SGP-1 in Sertoli cells was faint from

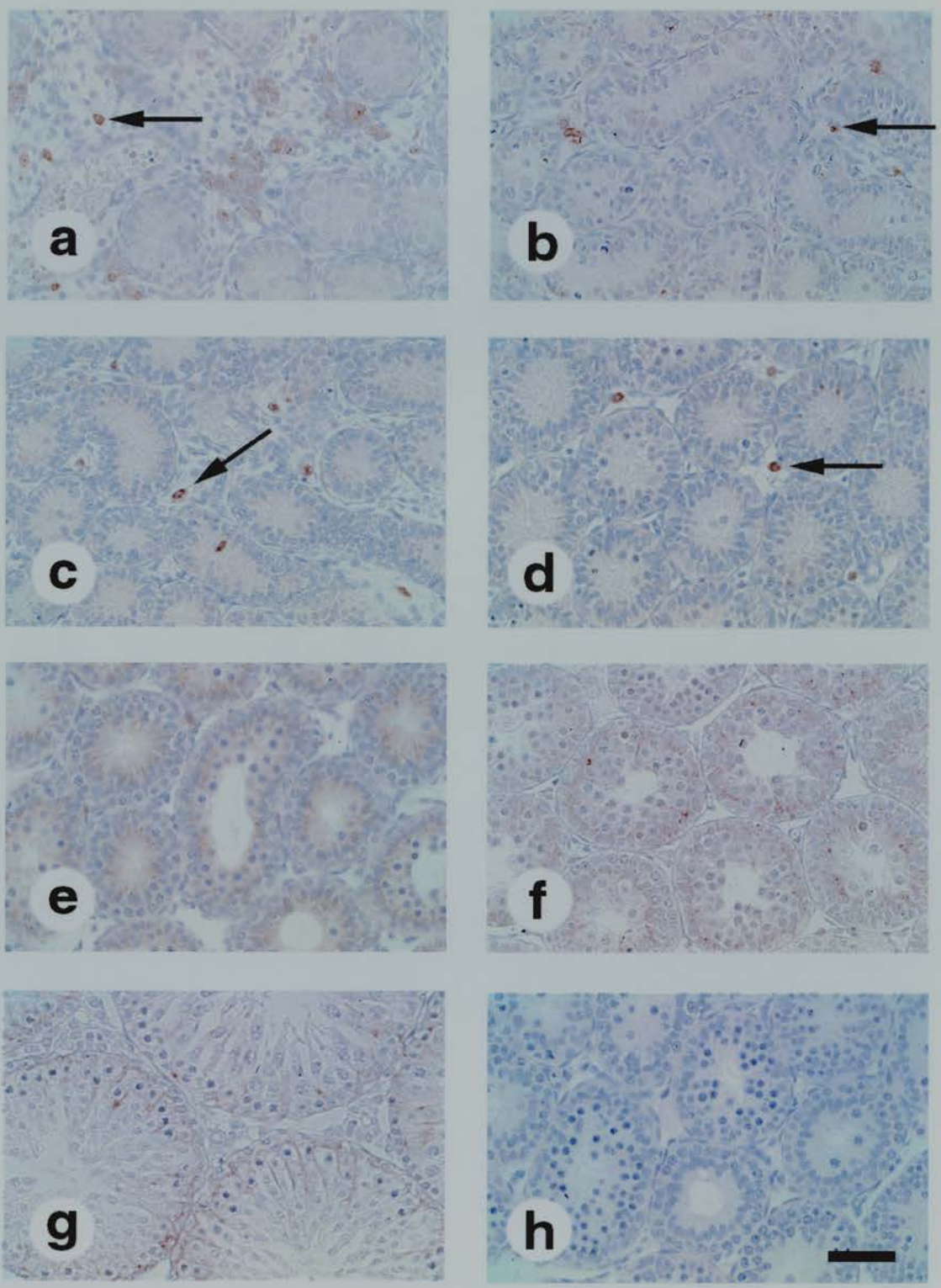

Figure 3.9: Immunolocalisation of SGP-1 to postnatal rat testis. Testes from rats aged fetal day 20.5 **(a)**, postnatal day 3 **(b)**, day 9 **(c)**, day 15 **(d)**, day 18 **(e)**, day 21 **(f)** and adult **(g)** are shown. A representative negative control using normal rabbit IgGs in place of primary antibody on day 18 testis is also shown **(h)**. Arrows point to immunopositive macrophages. Scale bar represents 50μm

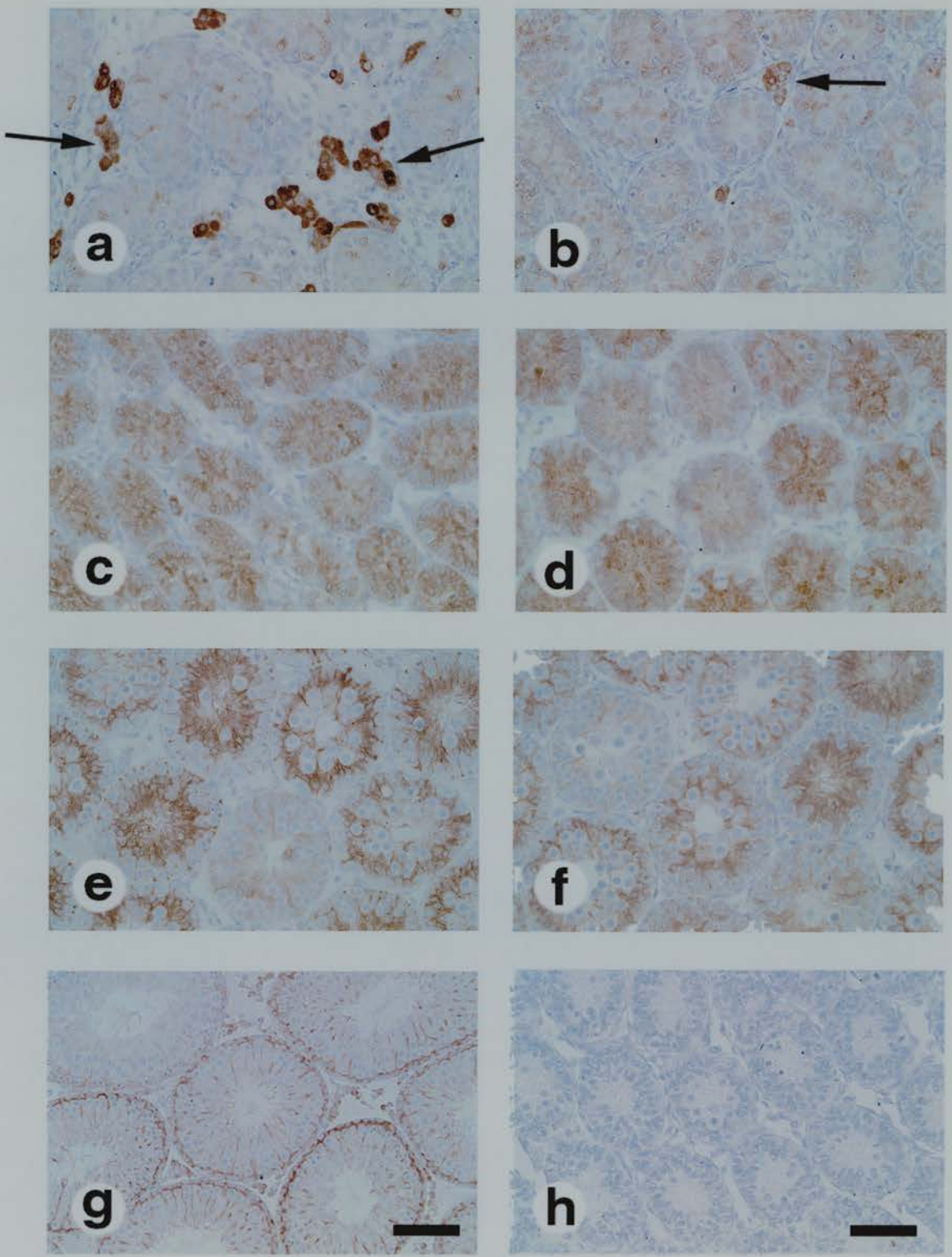

Figure 3.10: Immunolocalisation of inhibin-α to postnatal rat testis. Testes from rats aged fetal day 20.5 **(a)**, postnatal day 3 **(b)**, day 9 **(c)**, day 15 **(d)**, day 18 **(e)**, day 21 **(f)** and adult **(g)** are shown. A representative negative control using normal mouse IgGs in place of primary antibody on day 15 testes is also shown **(h)**. Arrows point to immunopositive interstitial cells which are thought to be fetal type Leydig cells. Scale bar (g) represents 100μM. Scale bar (h) represents 50μm.

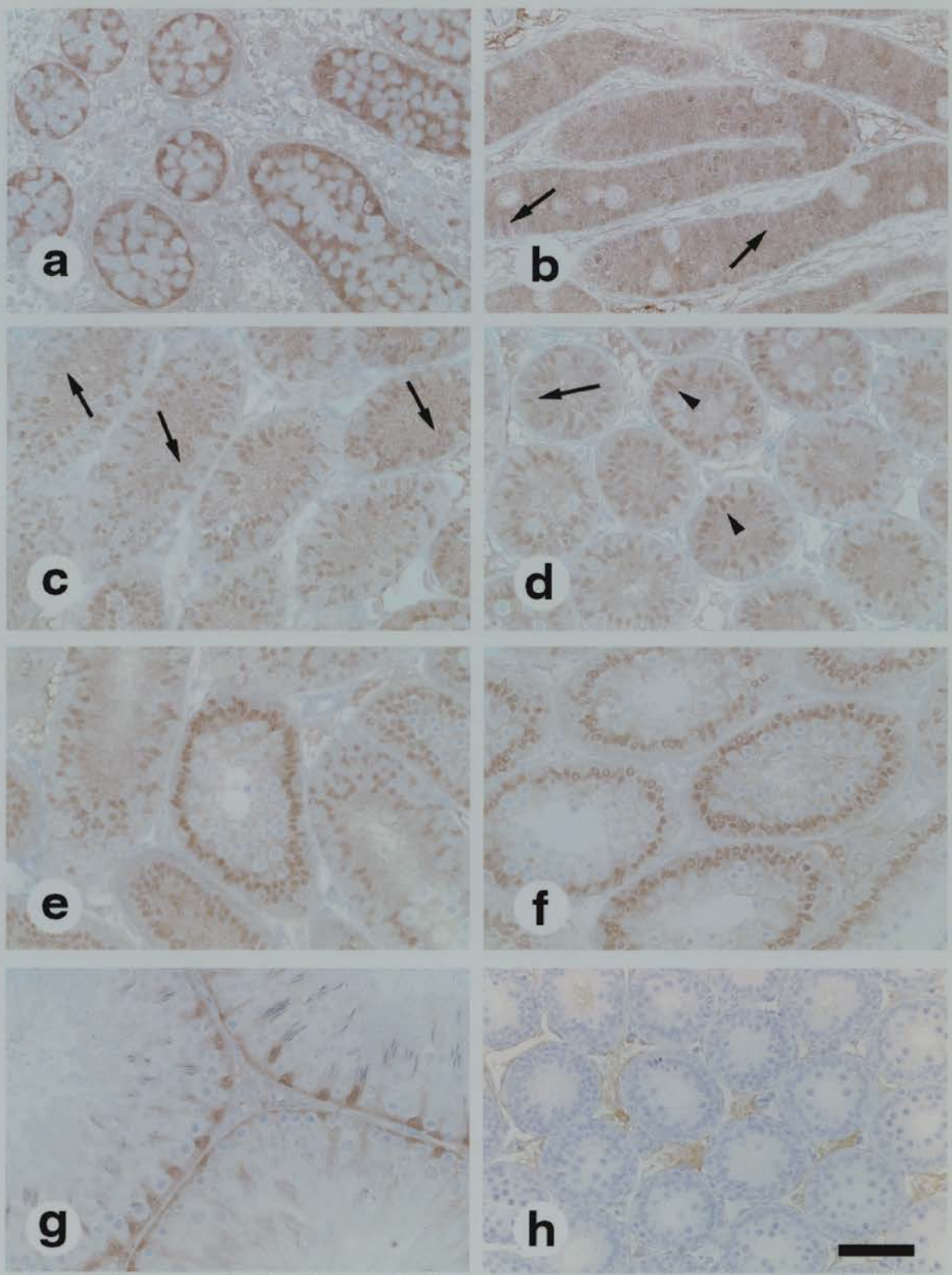

Figure 3.11: Immunlocalisation of Musashi-1 in postnatal rat testis. Testes from rats aged fetal day 17.5**(a)**,postnatal day 5**(b)**, day 11**(c)**, day 16**(d)**, day 19**(e)**, day 21**(f)** and adult**(g)** are shown. A representative negative control using normal rat IgGs on day 19 testis is also shown **(h)**. Arrowheads point to Sertoli cells with predominantly nuclear staining. Arrows point to Sertoli cells with equal nuclear:cytoplasmic immunolocalisation. Scale bar represents 50µm

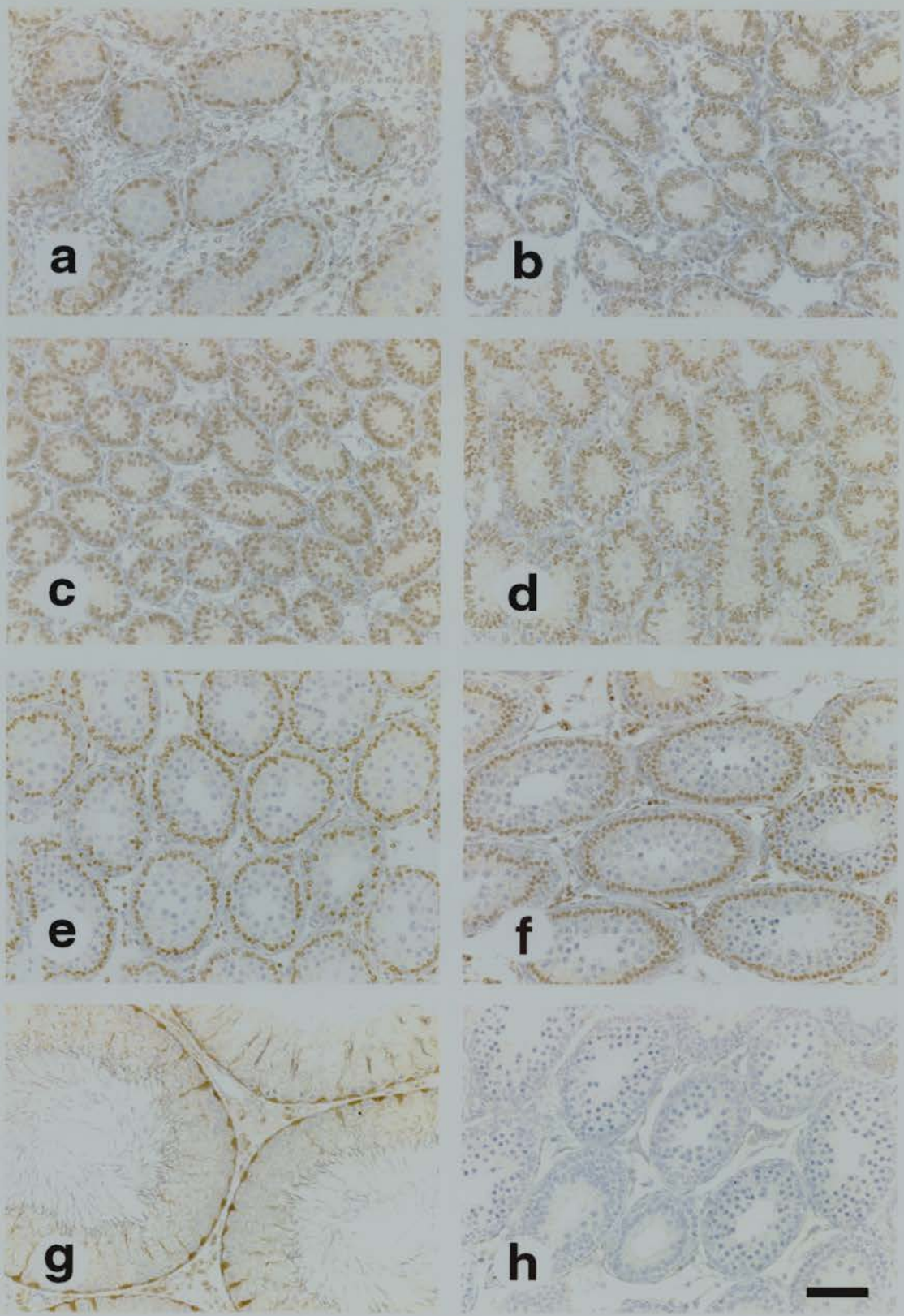

Figure 3.12: Immunolocalisation of GATA-4 in postnatal rat testis. Testes from rats aged fetal day 20.5 **(a)**, postnatal day 3 **(b)**, day 9 **(c)**, day 15 **(d)**, day 18 **(e)**, day 21 **(f)** and adults **(g)** are shown. A representative negative control using normal goat IgGs in place of primary antibody on day 18 testis is also shown **(h)**. Scale bar represents 50µm.

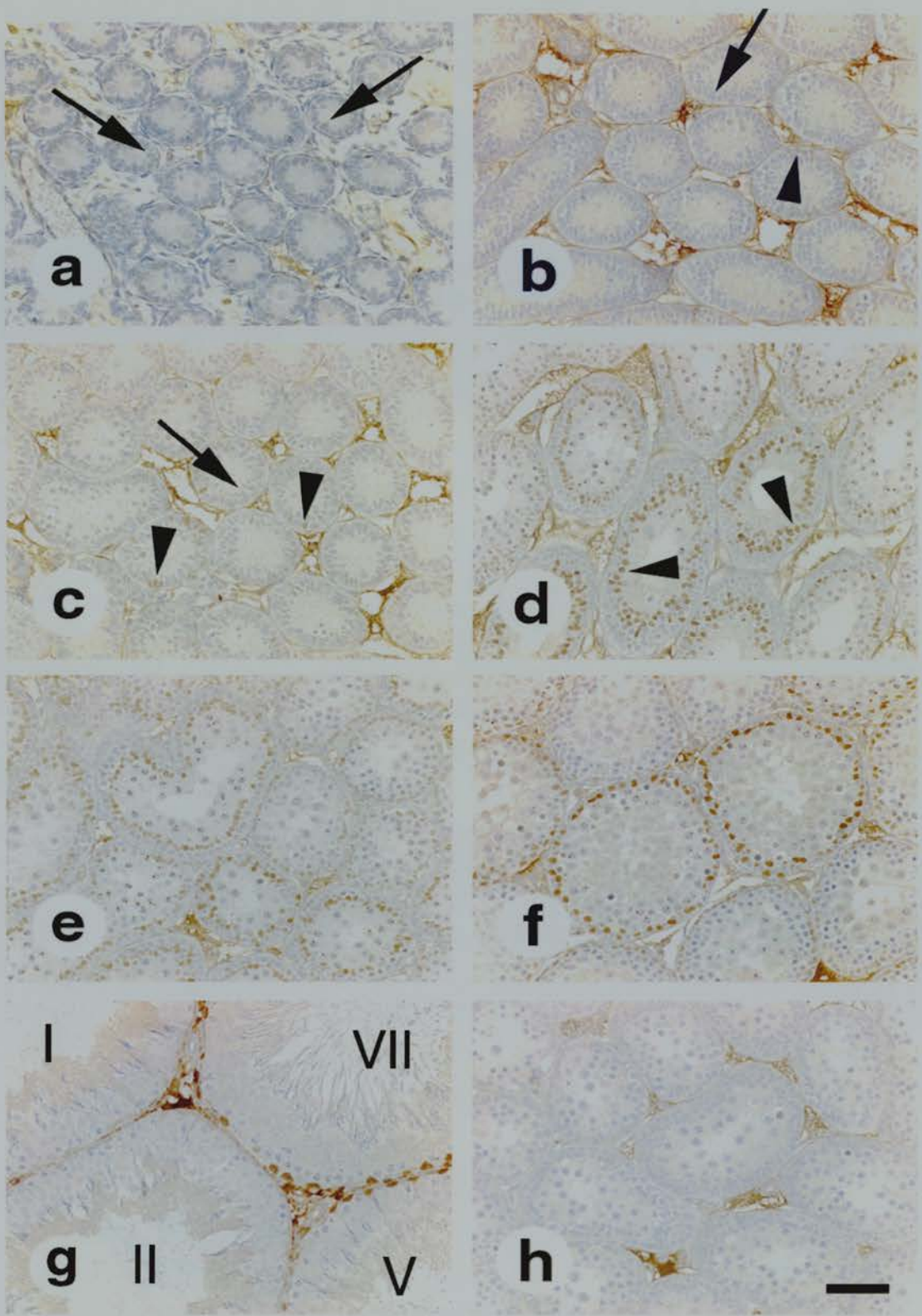

Figure 3.13: Immunolocalisation of GATA-1 in postnatal rat testis. Testes from rats aged postnatal day 3 **(a)**, day 14 **(b)**, day 15 **(c)**, day 18 **(d)**,day 21 **(e)**, day 28 **(f)** and adult **(g)** are shown. A representative negative control using normal rat IgGs in place of primary antibody on day 18 testis is shown **(h)** with non specific interstitial staining. Arrowheads point to immunopositive Sertoli cell nuclei. Arrows point to immunonegative Sertoli cell nuclei. Spermatogenic stages I, II, V and VII are shown in the adult testis (g). Scale bar represents 50µm.

fetal day 20.5 until day 15 (Fig. 3.9. a-d) after which the intensity of Sertoli cell immunostaining increased and by day 21 (Fig. 3.9.f) there was a strong immunopositive signal seen in all tubules and levels remained elevated into adulthood (Fig. 3.9.g).

3.3.3.2. Inhibin –α

Anti-inhibin-α antibody was localised to cytoplasm of interstitial cells in the fetal testis and in both fetal and postnatal Sertoli cells. In fetal day 20.5 testes (Fig 3.10.a) inhibin-α was strongly localised to the cytoplasm of interstitial cells: however the intensity of interstitial staining was reduced in day 3 testes (Fig 3.10.b). Between days 9 and 35 inhibin-α was not detected in interstitial tissue: however some interstitial cells were positively stained for inhibin-α in day 35 testes. Weak cytoplasmic Sertoli cell staining could be seen in fetal day 20.5 testis (Fig 3.10.a) and immunostaining was slightly upregulated by postnatal day 3 (Fig. 3.10.b) followed by a rapid upregulation in Sertoli cell inhibin-α expression between days 3 and day 9 (Fig 3.10.c). Inhibin-α immunostaining in all Sertoli cells remained strong up to day 15 when the intensity of staining became non uniform between tubules (Fig. 3.10.d). Variation in the intensity of immunostaining between Sertoli cells in tubules became more apparent between days 18 and 35 and inhibin-α expression was stage dependent in adults (Fig. 3.10.e-g).

3.3.3.3. Musashi-1

Using an anti-Musashi-1 antibody, strong immunolocalisation was observed in Sertoli cells at all ages examined. Some non-specific interstitial staining was also seen (Fig. 3.11.h). In fetal day 17.5 rat testes (Fig 3.11.a) there was intense immunopositive staining of both cytoplasm and nuclei which had decreased in intensity by postnatal day 5 (Fig. 3.11.b). At this time point staining was stronger in some Sertoli cell nuclei than in others. In day 11 and day 16 the number of Sertoli cells with prominent nuclear staining was increased whilst cytoplasmic staining remained uniform across all tubules (Fig.3.11.c, d). The majority of Sertoli cell nuclei in day 19 and day 21 testes had stronger nuclear

staining than cytoplasmic staining (Fig. 3.11.e, f) and in adult testes Musashi-1 nuclear staining was strongest in tubules at stages XI-VII. Cytoplasmic staining remained uniform across all stages (Fig. 3.11. g).

3.3.3.4. GATA-4

Strong immunolocalisation of antiserum directed against GATA-4 was restricted to Sertoli cell and interstitial cell nuclei in the rat testes. GATA-4 interstitial staining was observed in fetal day 20.5 and postnatal day 3 testes (Fig. 3.12.a, b) after which interstitial expression of GATA-4 was undetectable until day 18 (Fig. 3.12 e). Following day 18, interstitial cell expression of GATA-4 continued into adulthood (Fig. 3.12.g). Expression of GATA-4 in Sertoli cell nuclei was strong at all ages from fetal day 20.5 through to adulthood (Fig. 3.12.a-g). Expression of GATA-4 protein in Sertoli cells appeared to be upregulated after day 18.

3.3.3.5. . GATA-1

Specific immunolocalisation of anti-GATA-1 antibody was restricted to Sertoli cell nuclei. Non specific interstitial staining was observed in some testes (Fig. 3.13.h). Specific GATA-1 immunolocalisation was not observed in neonatal testis (Fig. 3.13.a). Weak GATA-1 nuclear staining was first observed in a very small number of Sertoli cells in day 14 testes (Fig. 3.13.b, arrowhead). GATA-1 expression was upregulated in day 15 testes (Fig. 3.13.c) and strong Sertoli cell nuclear immunostaining could be seen in all tubules by day 18 (Fig. 3.13.d). Between days 21 and 28 (Fig. 3.13.e,f) levels of GATA-1 expression varied between tubules. In the adult testes GATA-1 immunolocalisation was restricted to Sertoli cell nuclei in tubules at stages IV to VIII (Fig. 3.13.g).

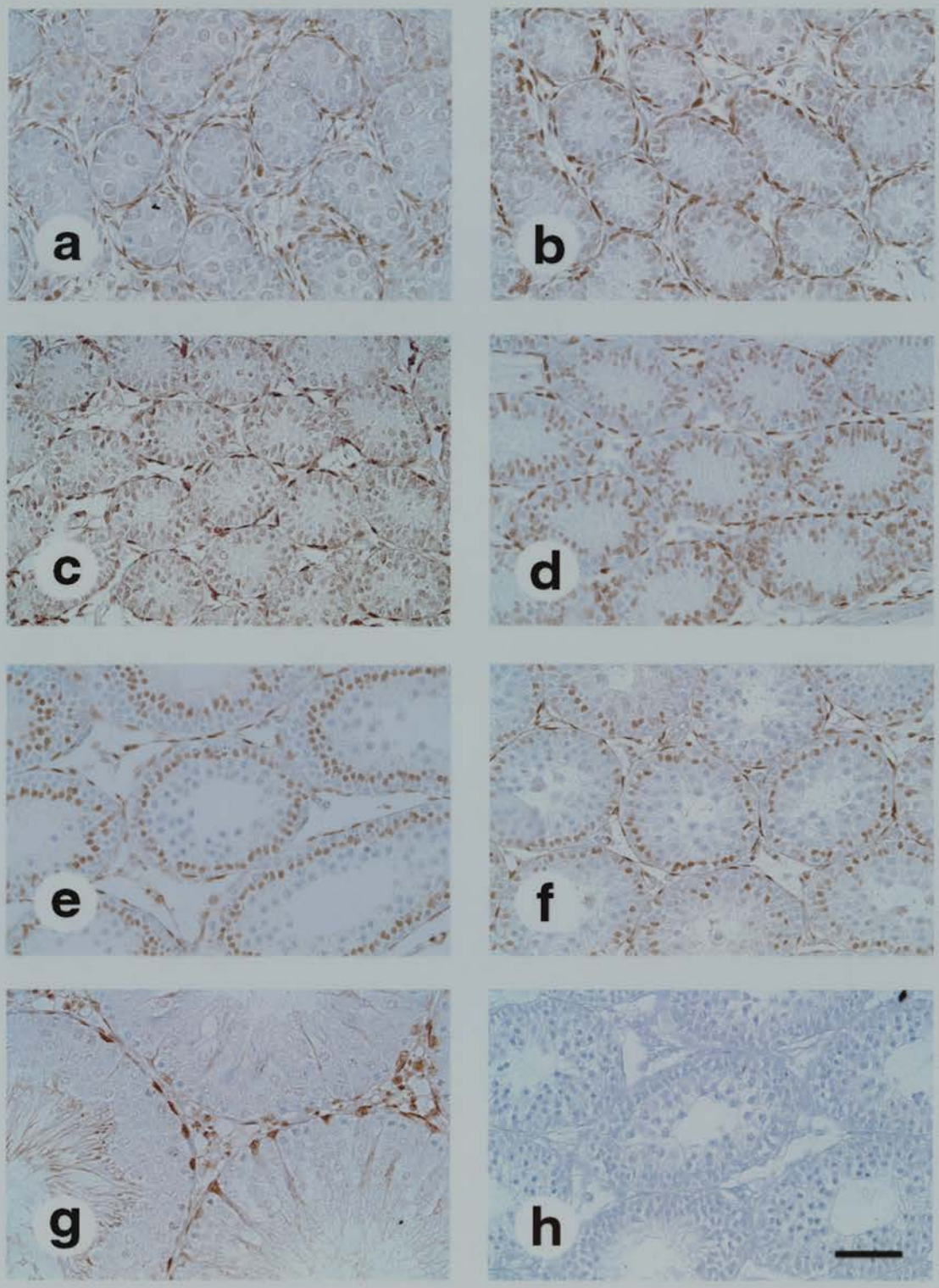

Figure 3.14: Immunolocalisation of androgen receptor in the postnatal rat testis. Testes from rats aged fetal day 20.5 **(a)**, postnatal day 3 **(b)**, day 9 **(c)**, day 15 **(d)**, day 18 **(e)**, day 21 **(f)** and adult **(g)** are shown. A representative negative control where normal rabbit IgGs replaced the primary antibody in day 21 testis is also shown **(h)**. Scale bar represents 50μm.

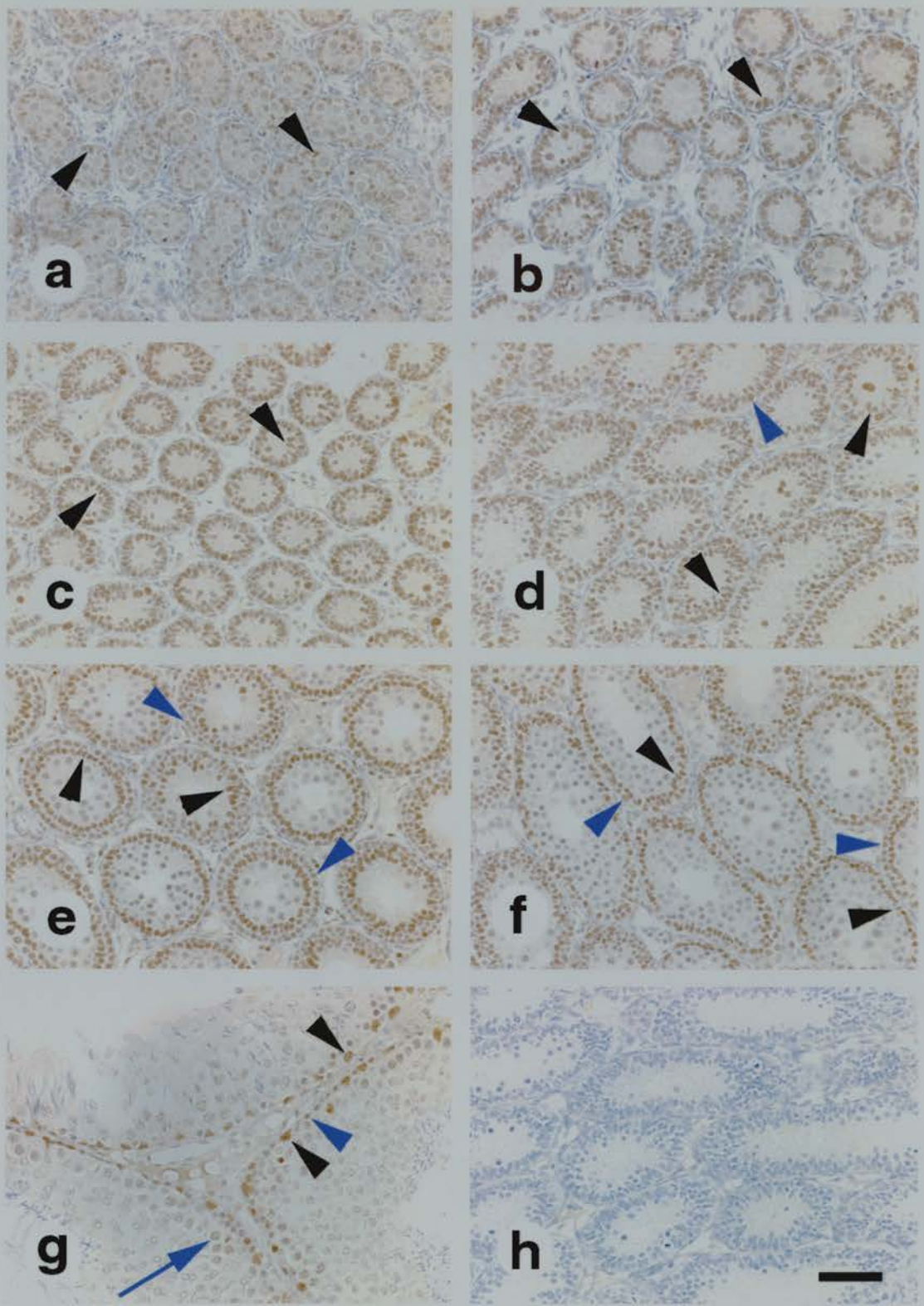

Figure 3.15: Immunolocalisation of oestrogen receptor-β in the postnatal rat testis. Testes from rats aged postnatal day 1**(a)**, day 3 **(b)**, day 9 **(c)**, day 15 **(d)**, day 18 **(e)**, day 21 **(f)** and adult **(g)** are shown. Immunostainng withpreabsorbed primary antibody on day 15 testis is also shown **(h)**. Black arrowheads point to immunopositive Sertoli cell nuclei, blue arrowheads point to immunopositive spermatogonia and blue arrows point to immunopositive early spermatocytes. Scale bar represents 50μm.

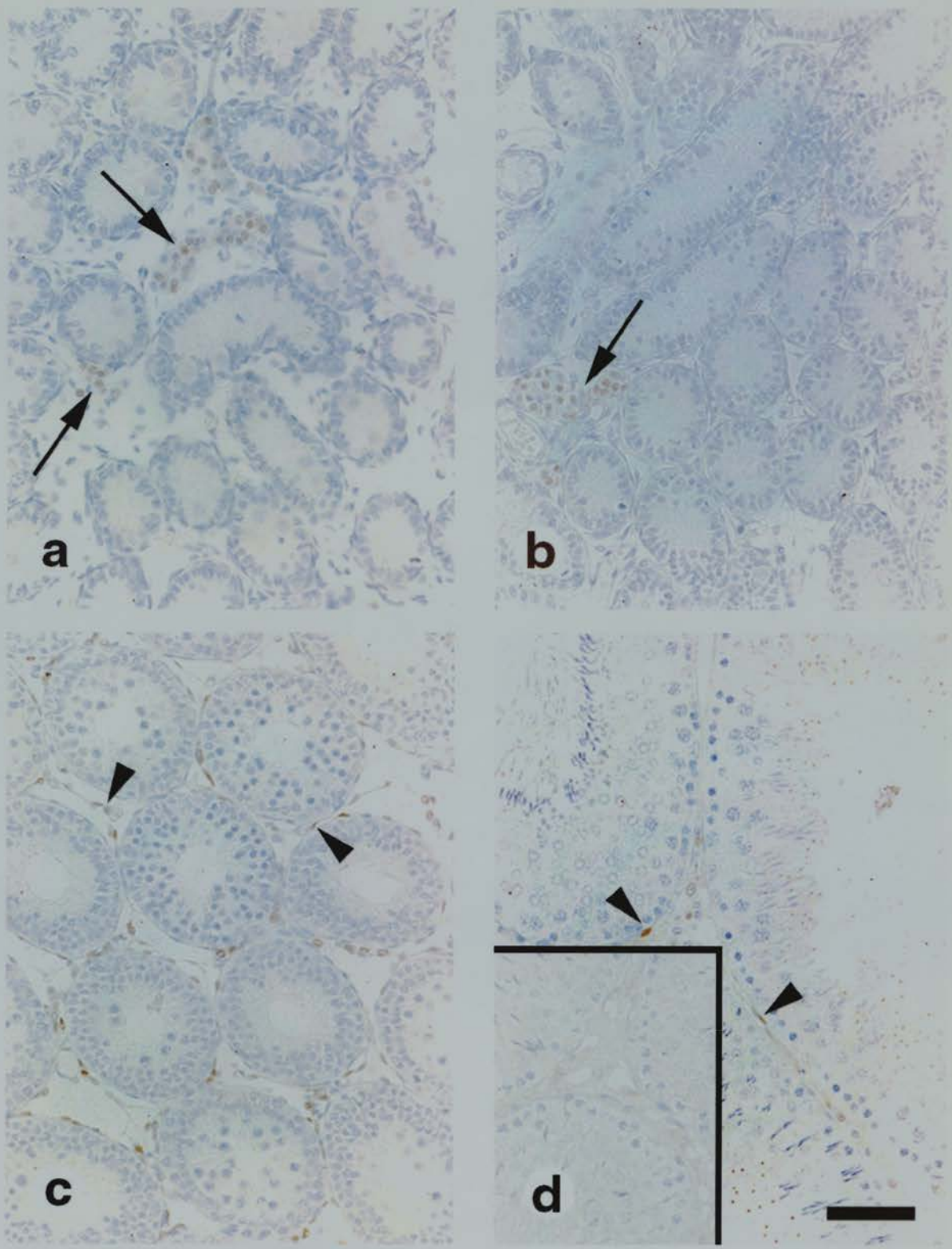

Figure 3.16: Immunolocalisation of oestrogen receptor-α in the postnatal rat testis. Testes from rats aged postnatal day 3 **(a)**, day 9 **(b)**, day 18 **(c)** and adult **(d)** are shown. Inset shows a representative negative control using normal mouse IgGs in place of primary antibody in adult testis is also shown. Arrows point to immunopositive fetal type Leydig cells. Arrowheads point to immunopositive adult type Leydig cells. Scale bar represents 50μm.

3.3.4. Immunoexpression of steroid hormone receptors

3.3.4.1. Androgen receptor (AR)

In the postnatal testis expression of AR protein was restricted to nuclei of Sertoli cells some interstitial cells and cells surrounding the outside of the tubules which were thought to be peritubular myoid cells. In fetal day 20.5 testis (Fig. 3.14.a) AR were localised to the nuclei of interstitial and peritubular myoid cells only. Between postnatal days 3 and 9 (Fig. 3.14.b, c) weak Sertoli cell AR immunostaining was observed and peritubular and interstitial cell staining remained strong. Sertoli cell AR immunoexpression was high in day 15 testes and levels of expression remained elevated through into adulthood where expression was stage specific (Fig. 3.13.d-g). The intensity of AR immunostaining in interstitial and peritubular myoid cells was high at all ages studied.

3.3.4.2. Oestrogen receptor beta (ERβ)

Using an anti-ERβ antibody, immunopositive staining was restricted to the nuclei of testicular cells. In day 1 testes (Fig.3.15.a) the intensity of ERβ Sertoli cell and gonocyte nuclear immunostaining was low. Between days 3 and 9 (Fig. 3.15.b, c) immunolocalisation of ERβ to Sertoli cells was upregulated (black arrowheads) whilst spermatogonial ERβ immunolocalisation remained low. After day 15, Sertoli cell nuclear ERβ staining had increased and ERβ expression levels remained high thereafter (Fig. 3.15.d-g). The intensity of ERβ immunostaining in spermatogonia was high from day 15 onwards (blue arrowheads) and low levels of expression in spermatocytes was present following day 21 (blue arrows). Interstitial staining was observed at all ages except in the adult.

3.3.4.3. Oestrogen receptor (ERα)

Immunolocalisation of an anti-ERα antibody was restricted to interstitial cell nuclei. Interstitial cells expressed ERα from postnatal day 3 through to adulthood (Fig. 3.16.a-d). Large clumps of positively immunostained cells could

be seen in interstitial tissue until postnatal day 9 (Fig. 3.16.a, b, arrows). By postnatal day 18 the majority of ERα immunostained nuclei were individually located throughout the interstitial tissue (Fig 3.16.c, arrowheads). This pattern of ERα immunoexpression remained into adulthood (Fig. 3.16.d).

3.4. Discussion

Development of the fetal testis into the mature adult testis is a highly organised and complex process. The modifications in cell proliferation, structure, morphology and function that take place during testicular postnatal development need to be tightly regulated and controlled. Due to the numerous paracrine interactions which are present within the testis at this time an alteration in the differentiation of one cell type can affect the functional maturation of the whole testis (De Franca *et al.*, 1995). In the present study, the development of the postnatal rat testis was investigated and selected 'markers' of Sertoli cell functional maturation were studied in detail. An alteration in Sertoli cell maturation either functionally (e.g. protein expression), morphologically (e.g. cytoplasmic extensions) or structurally (e.g. blood testis barrier formation) can affect the onset of spermatogenesis, the differentiation of adult type Leydig cells and tubule lumen formation (Bunick *et al.*, 1994; De Franca *et al.*, 1995; van Haaster *et al.*, 1992; van Haaster *et al.*, 1993) all of which might subsequently affect fertility in the adult.

During the present study, before maturational changes in the expression of Sertoli cell proteins were investigated it was deemed necessary to establish when Sertoli cells ceased dividing in the testis of the Sprague Dawley rats studied. During development testis weight is frequently used as a crude measure of growth and development. The changes in mean testes weight observed in the Sprague Dawley rats bred in our colony shown in Fig. 3.1 followed the same pattern of growth as shown in previous studies of rat testicular development (Kirby *et al.*, 1992; Meachem *et al.*, 1996; van Haaster *et al.*, 1993). The increases in testis weight in the present study were identical to the

increases in testis volume reported by Wang et al., (1989) and Zhengwei et al., (1990). PCNA and Ki67 immunolocalisation and BrDU incorporation suggested that the majority of Sertoli cell division had stopped between postnatal days 15 and 18 (Fig. 3.2-3.4). Thus the expansion in testis weight and volume which occurs after day 18 is due to the massive expansion in the germ cell population. In the present study primary spermatocytes were first detected in the rat testis on day 15 which is in agreement with previous reports (Clermont and Perey, 1957; Zhengwei *et al.*, 1990). Between birth and day 90 the volume density of the rat seminiferous tubules increases from 35.51% to 91.24% whilst absolute seminiferous tubule volume increases from 1.04mm^3 to 1238.42mm^3 at the same ages (Mendis-Handagama *et al.*, 1987). Thus the rapid expansion in testis weight after day 18 observed in the present study and in the literature is due to a massive increase in primary spermatocyte numbers after day 15 and the subsequent expansion in numbers of other germ cell types (Zhengwei *et al.*, 1990).

Immunostaining using an antibody directed against PCNA resulted in intense uniform, nuclear staining in the majority of cells in the testis from birth to day 21. Immunonegative cells were observed in the interstitial tissue at all ages studied and these cells may be non-proliferating Leydig cells. Immunonegative cells were also seen within the seminiferous tubules from day 9 onwards (Fig. 3.2.b). On day 9 immunopositive spermatogonia were present at the base of the tubules and the number of immunopositive spermatogonia had increased by day 18 (Fig. 3.2.e). The number of primary spermatocytes immunopositive for PCNA was increased by day 21 (Fig. 3.2.f) and this is in agreement with previously published results (Clermont and Perey, 1957; Zhengwei *et al.*, 1990). Staining of Sertoli cell nuclei was very intense in the neonatal testis (days 3-9): however by day 16 the majority of Sertoli cells were immunonegative for PCNA. A very small number of immunopositive Sertoli cells were present in day 21 testes which was a surprise as Sertoli cell proliferation is thought to cease by day 18 (Clermont and Perey, 1957; Orth, 1982), although Nagy, (1972) stated that rat Sertoli cells continued to incorporate 3H-thymidine until the 7th week of

postnatal life. Expression of PCNA protein is reported to be maximal during the transition from the G1 to the S phase during the normal cell cycle and lowest during the M phase (Celis and Celis, 1985; Kurki *et al.*, 1986). Mathews et al., (1984) have reported that PCNA is present continuously throughout mitosis. The biological half-life of PCNA is 20 hours (Bravo *et al.*, 1987) and therefore cells which have recently ceased to divide are likely to be immunopositive for PCNA. Hence immunopositive Sertoli cells in day 21 testis may have recently ceased proliferation but had remained immunopositive due to the long half-life of PCNA.

Due to the persistence of the PCNA protein in non-proliferating cells the same testicular series was examined using Ki67 immunolocalisation. The pattern of interstitial and germ cell Ki67 immunostaining was similar to that seen for PCNA except that the numbers of cells immunopositive for Ki67 were lower than for PCNA. This could be due to the fact that abundant expression of Ki67 is restricted to the G2 and M phases of the cell cycle with weaker Ki67 immunostaining in the S and late-G1 phases (Sasaki *et al.*, 1987; Wersto *et al.*, 1988). The biological half life of Ki67 is 1–2 hours: hence cells which have ceased proliferation will be immunonegative for Ki67 2 hours later (Duchrow *et al.*, 1994). The amount of Ki67 protein is at its lowest level immediately after mitosis and may not be detectable by immunohistochemical methods (Wersto *et al.*, 1988). This could not only explain the non-uniform staining pattern observed in Fig. 3.3 where some nuclei are stained more strongly than others but could also explain the lower number of mitotic cells which were detected using Ki67 compared to PCNA immunostaining. In comparison to PCNA immunolocalisation very few Sertoli cells were strongly stained for Ki67 in day 14 testis and by day 18 no positively stained Sertoli cells could be seen.

The thymidine analogue BrDU is incorporated into the DNA of cells only during the S phase of the cell cycle. In the present study the pattern of cells labelled with BrDU during the one hour between administration and tissue collection was similar to that reported previously (Meachem *et al.*, 1996; Sharpe *et al.*, 1999;

van Haaster *et al.*, 1993). Very few cells had intense BrDU immunolocalisation in day 15 testes. However some germ cells were immunopositive at this age. Low levels of BrDU labelling could result from low levels of BrDU incorporation due to DNA synthesis within these cells being almost complete when BrDU was administered. No immunopositive Sertoli cells could be seen in day 21 testes, BrDU labelling at this age was exclusively found in spermatogonia and primary spermatocytes and a few interstitial cells.

Immunolocalisation of PCNA, Ki67 and BrDU incorporated during the last hour prior to death, in the postnatal rat testis showed that the postnatal testicular development of the rats used in the present study agreed with patterns of development previously described (Clermont and Perey, 1957; Orth, 1982; Steinberger and Steinberger, 1971; Wang *et al.*, 1989) and that the majority of Sertoli cell proliferation occurs before day 10 and cessation of proliferation in most Sertoli cells takes place between days 15 and 18. A very small population of Sertoli cells may continue to proliferate until day 20 indicated by the perstitence of PCNA immunolocalisation in day 21 testis. The majority of spermatogonia and primary spermatocytes had entered cell division by day 15 and 18 respectively and germ cell populations expanded thereafter as indicated by the increase in testis weight observed at this time. Interstitial cell populations which included Leydig cells, replicated throughout the period of development studied. However non-proliferating cells were present in the interstitial tissue at all ages investigated.

Once we had established the age at which Sertoli cells stopped proliferating and the timing of the onset of spermatogenesis began in our rat colony expression, patterns of known and potential markers of Sertoli cell maturation were studied. Changes in mRNA expression were investigated using Northern blot analysis which provided an overview of changes in gene expression over the whole testis during development. In addition, using a phosphoimager and Imagequant programmes, hybridisation signals could be quantified enabling comparisons of mRNA expression over development to be carried out.

Immunocytochemistry was used to establish cellular localisation of proteins during development as well as enabling rough changes in protein levels of expression to be investigated.

The cDNA probe for rat SGP-1 detected a single 2.6kb transcript in testicular and kidney (not shown) RNA as described by Collard et al., (1988). The pattern of SGP-1 protein and mRNA expression observed in the present study was in agreement with previous reports (Mathur *et al.*, 1994).

SGP-1 immunopositive interstitial cells observed in the fetal and mature testis (days fetal 20.5 to postnatal day 15) are thought to be macrophages which are known to express SGP-1 (Morales *et al.*, 1996). Interstitial macrophages are individually distributed within the interstitial tissue in close proximity to Leydig cells (Niemi *et al.*, 1986). During postnatal development testicular macrophages are involved in the development and differentiation of adult type Leydig cells (Gaytan *et al.*, 1994) which begin maturation after day 15 (Mendis-Handagama *et al.*, 1987). Large quantities of SGP-1 protein may be required by macrophages during Leydig cell development. Once Leydig cells undergo differentiation the role of macrophages within the interstitial tissue could be modified resulting in a downregulation of SGP-1 protein synthesis. In fetal and neonatal testis some 'clumps' of SGP-1 positive cells were also found in the interstitium and their location suggested that they were likely to be the fetal population of Leydig cells.

To my knowledge there are no previous reports of SGP-1 protein expression in fetal type Leydig cells and the possible role of SGP-1 in these cells is unknown. Fetal type Leydig cells contain high levels of lipids and SGP-1 is involved in the transport and breakdown of glycolipids. Northern blot analysis of SGP-1 mRNA levels observed changes in total testis RNA extracts. Therefore the observed decline in SGP-1 mRNA expression after postnatal day 1 may be due to the decrease in function and number of fetal type Leydig cells (Mendis-Handagama *et al.*, 1987). Analysis of SGP-1 mRNA localisation in the postnatal

testis using in situ hybridisation techniques would need to be carried out in order to confirm SGP-1 expression in fetal Leydig cells.

Quantified 18S signals from Northern blot analysis were used to normalise mRNA signals in the present study such that changes in expression of factors across all cell types in the testes could be studied. Initially, quantified SGP-1 signals at each age were to be used in order to establish changes in Sertoli cell mRNA expression alone. However changes in SGP-1 expression which were observed when immunostaining and Northern blot analysis were undertaken meant that we felt the data might be compromised by using this marker as an internal standard and 18S rRNA values were therefore used. Values normalised against 18S reflect changes in levels of mRNA across the whole of the rat testis extract. In the present study assessment of testicular inhibin-α, Musashi-1 and GATA-4 mRNA levels showed decreases in mRNA levels after day 18; the age when germ cell populations are expanding. Thus Sertoli cell expression of the above factors may not be decreasing after day 18 but dilution of Sertoli cell mRNA transcripts in total testis RNA extracts by increasing germ cell mRNAs maybe taking place.

Expression of inhibin–α mRNA in total testicular extracts reached maximal levels on day 15. The slight decrease in inhibin-α expression observed in day 18 testis is thought to result from the high background signal surrounding the actual 18S signal in the day 18 RNA sample in Fig. 3.6.B resulting in over estimation of the amount of RNA loaded. There is general agreement in previously published studies and the present study that levels of inhibin–α mRNA in total testis extracts increase immediately after birth. In the present study expression of inhibin-α declined after day 21 and inhibin-α mRNA levels in the adult testes were approximately 33% of levels in the testes at birth. This was in agreement with published changes in inhibin-α mRNA levels during postnatal development (Keinan *et al.*, 1989; Tena-Sempere *et al.*, 1999). Inhibin-α expression levels in whole testis RNA extracts are therefore maximal at the time that Sertoli cell proliferation ceases.

The observed developmental pattern of inhibin-α protein expression in both Sertoli and Leydig cells was similar to that described in the literature (Majdic *et al.*, 1997; Noguchi *et al.*, 1997; Rivier *et al.*, 1988). Changes in plasma levels of inhibin-α also reflect the pattern of changes in protein levels observed by immunocytochemical staining. Inhibin-α plasma levels are highest at day 14 and then decline with increasing age (Maddocks and Sharpe, 1990; Rivier *et al.*, 1988; Ultee-van Gessel and de Jong, 1997). Stage dependent expression of inhibin–α is first observed in day 18 testis and it is around this time that pachytene spermatocytes are first observed in the seminiferous tubules (Clermont and Perey, 1957). Levels of inhibin-α mRNA and protein are increased in tubules which lack pachytene spermatocytes (Kaipia *et al.*, 1991). Thus the onset of stage specific inhibin-α expression in Sertoli cells may be due to the appearance of pachytene spermatocytes which then inhibit Sertoli cell inhibin-α expression.

Novel results were found in the expression pattern of Musashi-1 mRNA during postnatal development and were different from those seen in a preliminary study by Maguire et al., (1999). In the initial study levels were maximal in day 6 testes and declined thereafter. However in this study Musashi-1 mRNA expression was maximal at day 18 and then levels declined. In the present study Musashi-1 mRNA was normalised against 18S values which therefore showed changes in expression over the whole testis. Musashi-1 expression in the testis is restricted to Sertoli cells (see Fig. 3.11). Therefore any changes in Sertoli cell Musashi-1 expression between different ages would still be apparent. Maguire et al (1999) (Maguire *et al.*, 1999) used SGP-1 mRNA levels to normalise Musashi-1 mRNA signals. This would also show any changes in specific Sertoli cell Musashi-1 expression. However SGP-1 mRNA levels are themselves changing over this period of time (see Fig. 3.4.C). This may account for the discrepancies in Musashi-1 expression observed between the two studies.

After birth, Musashi-1 mRNA levels increase. However there is no simultaneous increase in protein levels observed at this time. It is possible that

Musashi-1 mRNA transcripts have a short half life in the proliferating Sertoli cell such that in order to maintain functional levels of Musashi-1 protein an upregulation in mRNA expression is required. On the other hand Musashi-1 protein could have a short half life such that an increase in protein levels during this period of development is not detected by immunocytochemistry. Musashi-1 expression in Sertoli cells has only recently been reported and consequently very little research has been carried out regarding patterns of expression or regulation of Musashi-1 in the Sertoli cell. Musashi-1 is used as a marker of undifferentiated and proliferating neuronal cells and Musashi-1 expression is lost following neuronal cell differentiation (Kaneko *et al.*, 2000). The increase in nuclear immunolocalisation of Musashi-1 detected in both Maguire et al., (1999) and this study, is maximal at the onset of Sertoli cell differentiation. This indicates that the role of Musashi-1 in Sertoli cells may be modified as the cell switches from a proliferating undifferentiated cell to a morphologically and functionally mature cell. Musashi-1 may be involved in nuclear-cytoplasmic RNA trafficking in mitotic Sertoli cells but becomes predominantly involved in RNA processing within the nucleus during Sertoli cell differentiation. In the adult testis nuclear localisation of Musashi-1 protein is maximal in tubules at stages with low testosterone levels (Maguire *et al.*, 1999) which suggests the possibility of steroidal regulation of Musashi-1 expression. However further research is necessary to confirm this.

The expression pattern of GATA-4 during postnatal development were similar to that already reported in the literature. In the present study GATA-4 mRNA levels increased steadily after birth reaching maximal levels between days 11 and 18 after which levels rapidly decreased to low levels which were observed in the adult testes. Ketola et al (1999) saw a similar pattern of expression in the mouse postnatal testis. However Viger et al., (1998) saw maximal levels of GATA-4 mRNA between birth and day 14. In all three studies a decline in GATA-4 mRNA was concurrent with Sertoli cell maturation and the appearance of germ cells. Possible reasons for the differences between this study and that

of Viger et al., (1998) include differences in GATA-4 expression between different strains of rat.

Studies on the expression of GATA-4 in the postnatal testis were extended to the protein and cellular level using immunocytochemistry. GATA-4 protein was strongly expressed in Sertoli cell nuclei through out postnatal development and expression levels remained high in the adult. Interstitial staining was also strong in fetal day 20.5 testes, levels decreased slightly during neonatal development but increased again in day 18 testes and expression remained high in adult interstitial tissue. GATA-4 protein has been previously shown to be expressed in Leydig cells (Ketola *et al.*, 1999) and thus the interstitial cell population that was immunopositive for GATA-4 in this study were thought to be Leydig cells.

It has been previously stated that GATA-4 mRNA expression is restricted to germ cells in the adult mouse and rat testis (Arceci *et al.*, 1993; Viger *et al.*, 1998). GATA-4 protein expression in germ cell nuclei was not observed in this study using rat testes nor was it observed in germ cells in mouse postnatal testes by Ketola et al., (1999). Identical primary antibodies at the same concentrations were used in all three studies and the same immunocytochemical protocols were carried out except that different fixation techniques were used to those in Viger et al., (1998) and frozen tissue sections were used by Ketola et al., (1999). Further research is necessary to confirm the expression of GATA-4 in the differentiated Sertoli cell.

GATA-4 expression is essential for the proliferation and differentiation of cardiomyocytes (Grepin *et al.*, 1995). Granulosa cells in early follicles have strong GATA-4 expression up until ovulation and GATA-4 levels then decline with luteinization of the follicle (Heikinheimo *et al.*, 1997). It has been suggested that GATA-4 acts as a survival transcription factor in cellular development and differentiation. In the present study GATA-4 was expressed throughout Sertoli cell development, differentiation and maturation and could therefore act as a survival transcription factor in Sertoli cells. GATA-4 expression does not

however appear to change with increasing Sertoli cell maturation as has been previously reported and is therefore not an ideal marker for Sertoli cell functional maturation.

Analysis of GATA-1 mRNA expression over the postnatal period of development was investigated using both Northern blot analysis and Ribonuclease Protection Assays (RPA). However using both techniques no GATA-1 transcripts could be detected at any age or in the control spleen RNA sample (not shown). The GATA-1 cDNA probe which was used in the above analysis was originally amplified using PCR from a pool of cDNA species from day 21 rat testis created using oligo dT primers. Thus GATA-1 mRNA is expressed in day 21 rat testis. RTPCR was not used to determine changes in GATA-1 mRNA expression over the postnatal period as although RTPCR is a more sensitive technique, accurate quantification of RTPCR results could not be carried out and hence Northern blot and RPA analysis were subsequently attempted.

Changes in spatial and temporal GATA-1 protein expression were investigated using immunocytochemistry. GATA-1 protein could not be detected in neonatal testes however low levels of GATA-1 protein were detected in the nuclei of a small population of Sertoli cells in day 14 testes. GATA-1 protein levels increased thereafter and expression was becoming stage specific by day 18 testes. In the adult testis GATA-1 protein expression is restricted to Sertoli cell nuclei in tubules at stages IV to VIII.

The chronology of GATA-1 protein expression in the testis observed in this study is in good agreement with reported patterns of GATA-1 mRNA (Beau *et al.*, 2000; Onodera *et al.*, 1997; Viger *et al.*, 1998) and protein expression (Beau *et al.*, 2000; Viger *et al.*, 1998; Yomogida *et al.*, 1994) in rat and mouse testes. In adult mouse testes GATA-1 protein localisation was restricted to stages VI-IX (Yomogida *et al.*, 1994) whilst stage specific staining in the present study was very low in stage IV and was then restricted to stages V-VIII. These differences

in stage specific expression may result from species differences in GATA-1 expression between mice and rats. Moreover the staging of spermatogenesis is slightly different between the two species; in the mouse, spermatogenesis is divided into 12 stages compared to 14 stages in the rat testis. The stage dependent expression of GATA-1 is thought to result from inhibition of GATA-1 expression by germ cells (Yomogida *et al.*, 1994). GATA-1 is involved in a negative autoregulatory loop such that in immortal Sertoli cell lines GATA-1 can bind to its own promoter region and specifically repress its own transactivation, This is also thought to contribute to the stage specific expression of GATA-1 (Martin and and Orkin, 1990; Onodera *et al.*, 1997; Whyatt *et al.*, 1997).

GATA-1 expression is essential for the survival and cell differentiation of erythroid cell populations. (Heikinheimo *et al.*, 1997). The increase in GATA-1 protein expression is concomitant with the cessation of Sertoli cell proliferation and the start of Sertoli cell maturation. Thus it has been suggested that GATA-1 may act as a survival factor in non proliferating Sertoli cells and is involved in the transcription of genes expressed during cell maturation and differentiation as well as regulating the expression of genes involved in the onset and continuance of spermatogenesis.

In the present study Sertoli cell expression of SGP-1, inhibin-α and GATA-1 proteins was upregulated at the time of Sertoli cell differentiation. Immunolocalisation of Musashi-1 in Sertoli cell nuclei became more abundant with the increased maturation of the Sertoli cell. Therefore in future studies these factors will be used as markers of Sertoli cell functional maturation.

In the final part of this study immunolocalisation of ERα, ERβ and AR during testicular development was investigated so that possible sites of direct steroid action within the postnatal testis could be established. The expression pattern of AR observed in the present study was in agreement with previously published work (Bremner *et al.*, 1994; Buzek and Sanborn, 1988; Majdic *et al.*, 1995; Sharpe *et al.*, 1998). AR was expressed in interstitial and peritubular myoid cells

throughout postnatal development whereas AR expression was only detected in Sertoli cell nuclei from postnatal day 3 onwards. Levels of AR in the Sertoli cell increased with increasing age and became stage dependent between day 21 and adulthood. No AR expression was observed in any germ cells at any of the ages studied. Expression of ERβ protein during postnatal testes development corresponded well with published reports on ERβ expression in the testis (Saunders *et al.*, 1998; van Pelt *et al.*, 1999). From birth through to adulthood ERβ was strongly expressed in Sertoli cell nuclei. ERβ expression was also observed in gonocytes, spermatogonia and pachytene spermatocytes as well as in interstitial cells which were thought to be fetal and immature Leydig cells. In contrast, testicular ERα expression was restricted to Leydig cells. Both fetal and adult type Leydig cells expressed ERα throughout postnatal development which was also observed in Fisher et al., (1997).

Therefore both fetal and adult type Leydig cells express all three steroid receptors throughout rat postnatal development. Prespermatogonia, spermatogonia and early spermatocytes strongly express ERβ during postnatal life. Sertoli cells express ERβ throughout postnatal development and AR from the third day of life. Consequently during postnatal rat testis development both testosterone and oestrogen can directly affect Sertoli cells. In future studies the effect of both steroids on Sertoli cell maturation during postnatal development will be investigated using the Sertoli cell proteins assessed in the present study as indicators of modified Sertoli cell functional maturation.

Chapter 4.

The effect of *in vivo* manipulation of oestrogen levels on Sertoli cell gene expression.

4.1. Introduction

The function of oestrogen in the male reproductive tract, in particular the role of oestrogen during the development and maturation of the testis, has become the subject of considerable research activity. This interest has been stimulated by the proposed link between increases in the incidence of male reproductive abnormalities in humans and other wildlife and increased levels of oestrogen and oestrogen like chemicals in the environment (Sharpe and Skakkebaek, 1993; Toppari *et al.*, 1996). It is notable that fetal/neonatal exposure of rodents to high levels of oestrogen cause a range of abnormalities of the male reproductive system including small testis and delayed puberty (Arai *et al.*, 1983; Gaytan *et al.*, 1986; Newbold and McLachlan, 1985; Toppari *et al.*, 1996). It was initially suggested that these abnormalities were indirectly caused via suppression of gonadotropin release from the anterior pituitary by the elevated oestrogen levels (Gharib *et al.*, 1990). It was proposed that the net result of decreased levels of FSH was a delay in the onset of puberty and a reduced rate of Sertoli cell proliferation (Cunningham *et al.*, 1978; Meachem *et al.*, 1996) which in turn lowered the sperm output of the adult testis (Bellido *et al.*, 1990).

Male ERKO transgenic mice which express inactivated ERα protein are infertile. This finding not only illustrates that oestrogens act directly within the male reproductive tract but demonstrates an essential role for oestrogen during normal male reproductive function (Eddy *et al.*, 1996). ERα expression within the testis is restricted to Leydig cells (Fisher *et al.*, 1997): however the β form of the oestrogen receptor, ERβ, has recently been localised to Sertoli cells as well as germ cells throughout development and thus provides a direct pathway for oestrogen action in the developing Sertoli cell (Saunders *et al.*, 1998; van Pelt *et*

al., 1999). Direct action of oestrogens on Sertoli cell maturation and function has been investigated by Sharpe et al., (1998). Neonatal administration of oestrogen to male rats not only resulted in a delay in Sertoli cell maturation within the peripubertal testis but also had a permanent adverse effect on Sertoli cell function in the mature testis. It is notable that the results obtained suggest that these changes were not caused by suppression of FSH levels alone.

In Chapter 3 the expression pattern of GATA-1 and Musashi-1 proteins in Sertoli cells were found to be altered with increased Sertoli cell differentiation. Heikinheimo et al., (1997) have observed an upregulation of GATA-4 expression in granulosa cells following treatment with DES. In addition, FSH upregulates GATA-4 mRNA expression in MSC-1 Sertoli cells transfected with FSH receptor (Heikinheimo *et al.*, 1997). In erythroid cells ERα forms ligand–dependent protein:protein interactions with GATA-1 protein which subsequently inhibits GATA-1 transactivation of essential survival and differentiation genes (Blobel and Orkin, 1996; Blobel *et al.*, 1995). Although ERα is not expressed in Sertoli cells it is possible that ERβ could form protein associations with GATA-1 or GATA-4 in the postnatal Sertoli cell and suppress transactivation of GATA-1/4 regulated genes which are thought to include inhibin-α, aromatase, AMH, FSH receptor and GATA-1 itself (Beau *et al.*, 2000; Feng *et al.*, 1998; Martin and and Orkin, 1990; Onodera *et al.*, 1997; Whyatt *et al.*, 1997).

The present study made use of tissue sections which had been used previously in Sharpe et al., (1998). The effect of in vivo neonatal oestrogen administration on the immunoexpression patterns of GATA-1, GATA-4 and Musashi-1 in the rat testis has not previously been investigated. In the present study the effect of oestrogen on functional maturation of Sertoli cells was further investigated by assessing changes in expression of GATA-1 and Musashi-1 proteins during Sertoli cell differentiation. It had already been established that this treatment regime caused a maturational delay in Sertoli cell development and it was therefore hoped that a change in the expression patterns of the above factors would be observed hence confirming their use as markers of Sertoli cell

maturation. In addition the possible regulation of GATA-1, Musashi-1 and GATA-4 protein expression by oestrogen in the postnatal Sertoli cell could be studied for the first time.

4.2. Materials and Methods

Rat testes used in the present study had been originally collected for a separate study by Dr. Richard Sharpe the results of which have been published (Atanassova *et al.*, 1999; Sharpe *et al.*, 1998).

4.2.1. Animals and treatments

The experimental set up and treatment of animals was kindly carried out by Dr. Richard Sharpe and details are described in Sharpe et al., (1998) (Sharpe *et al.*, 1998) and Atanassova et al., (1999). Briefly, male Wistar rats were administered by subcutaneous injection either 10μg of DES (Sigma) in 20μl of corn oil, 10μg of ethinyl oestradiol (EE) in 20μl of corn oil or 20μl of corn oil (control group) on postnatal day 2, 4, 6, 8, 10 and 12. On day 18 or day 25 rats were killed as described in section 2.and testes were removed, fixed and processed as described in Chapter 2.

4.2.2. Immunocytochemistry

Testes from control animals and both treatment groups were placed on the same slide and were processed in parallel. Testes from a minimum of three different animals per group and from two separate experiments were analysed.

Immunolocalisation of inhibin-α, Musashi-1, GATA-4 and GATA-1 to fixed testes sections was performed as in section 3.2.6.2. A monoclonal antibody against PCNA was applied to fixed testes sections as described in section 3.2.6.1.
Sections were analysed using an Olympus Provis microscope and images were captured onto computer using either a Kodak DCS420 camera or a Kodak DCS330 camera and images were stored on the computer using the Adobe Photoshop 5.0 programme.

4.3. Results

The delay in the onset of spermatogenesis in day 18 testes of DES and EE treated animals suggested that postnatal testicular development had been modified; the delay in testicular development was most notable in testes from DES -treated animals. Immunoexpression patterns of all five proteins (inhibin-α, GATA-4, Musashi-1, GATA-1 and PCNA) in control day 18 and day 25 testes were identical to those reported in chapter 3. Following either neonatal DES or EE treatment no stage dependent inhibin-α immunoexpression could be observed in day 18 testes (Fig. 4.1.d, g). However there was no change in GATA-4 immunoexpression in day 18 testes of DES and EE treated rats when compared to control rat testes (Fig. 4.1.b, e, h). Stage dependent immunolocalisation of Musashi-1 was observed in day 18 control testes but not observed in either DES or EE treated testes. Nuclear immunolocalisation of Musashi-1 was stronger in EE treated testes than in DES treated testes where nuclear and cytoplasmic immunostaining were of similar intensity (Fig. 4.1.f, i).

In Figure 4.2 no GATA-1 nuclear immunolocalisation could be observed in the day 18 testes following neonatal DES treatment (Fig. 4.2. b, e). Similar levels of strong GATA-1 immunoexpression could be seen in Sertoli cell nuclei of both EE treated and control day 18 rat testes (Fig. 4.2 a, c, d, f). Sertoli cell nuclei were immunopositive for GATA-1 in both control and neonatal DES treated day 25 rat testes (Fig. 4.2. g-j). GATA-1 immunoexpression in Sertoli cell nuclei was becoming stage specific in day 25 DES treated testes and looked similar to the pattern of immunoexpression in day 18 control and EE treated testes.

Intense immunostaining for PCNA was localised to spermatogonia, Sertoli cells and Leydig cells in day 18 testes. In DES treated testes the majority of Sertoli cell nuclei were immunopositive in contrast to testes from control and EE

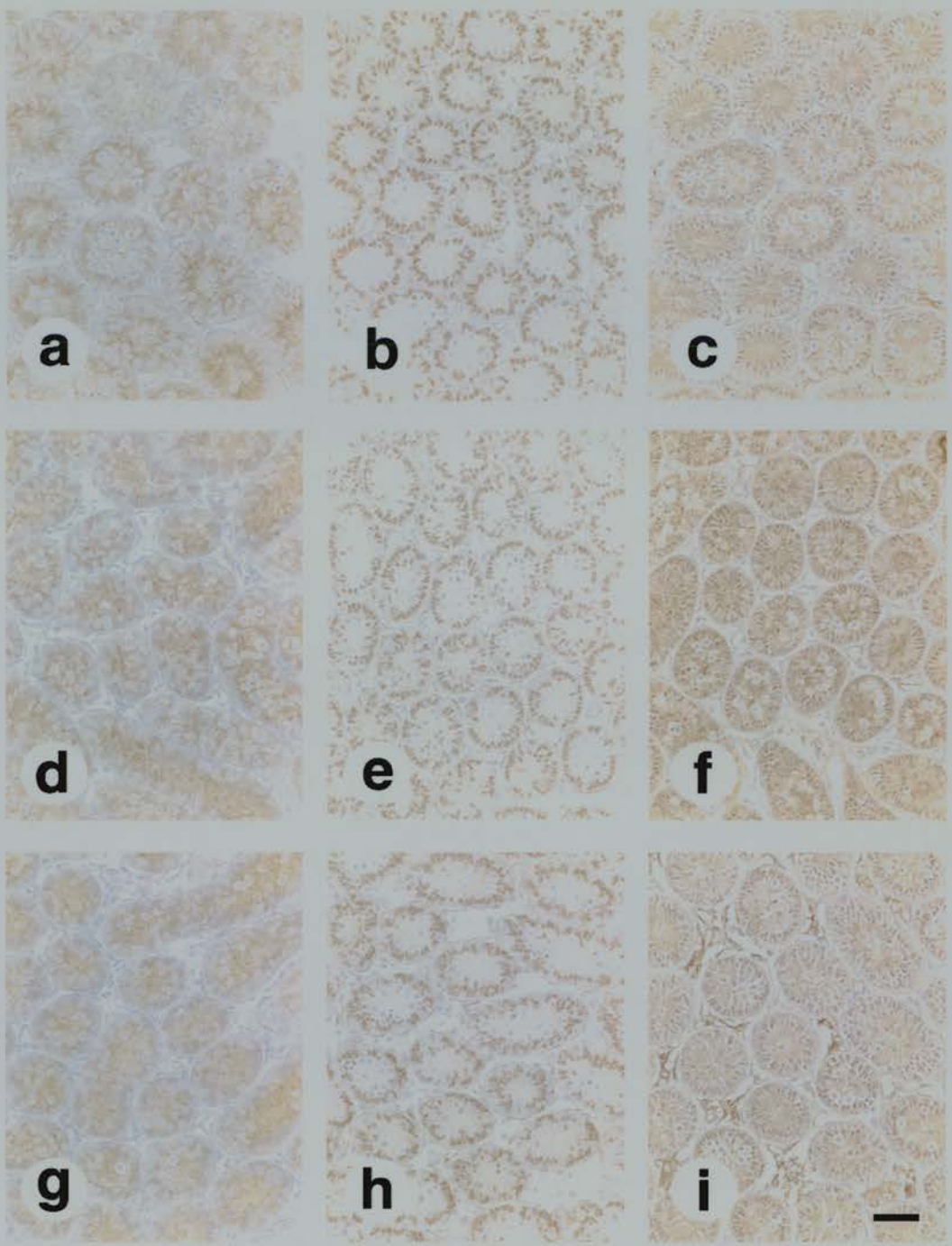

Figure 4.1: Immunolocalisation of markers of Sertoli cell functional maturation
Immunolocalisation of inhibin-α (**a, d, g**), GATA-4 (**b, e, h**) and Musashi-1 (**c, f, i**) to day 18 postnatal rat testes following neonatal treatment with DES-10μg (**d, e, f**), EE-10μg (**g, h, i**) and corn oil (**a, b, c**). Representative negative controls can be seen in Figures 3.10-12. Scale bar represents 50μm.

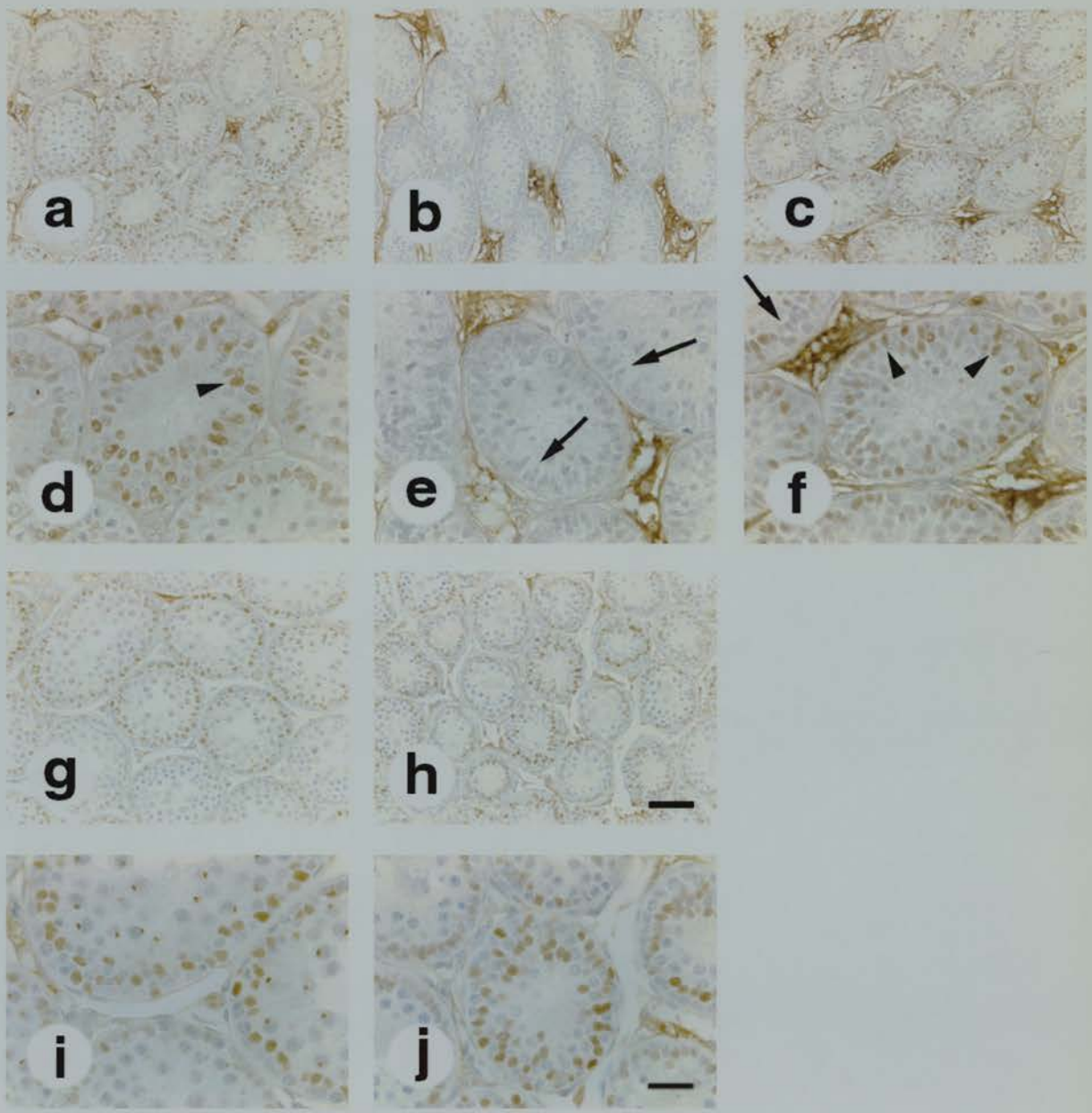

Figure 4.2: Immunolocalisation of GATA-1 to postnatal rat testes following neonatal treatment with DES (10μg) and EE (10μg). Testes from rats aged postnatal day 18 **(a-f)** are shown following neonatal treatment with DES-10μg **(b, e)**, EE-10μg **(c, f)** and corn oil **(a, d)**. Testes from rats aged postnatal day 25 **(g-j)** are shown following neonatal treatment with DES-10μg **(h, j)** and corn oil **(g, i)**. Images a-c, g, h were taken at X400 magnification and scale bar **(h)** represents 50μm. Images d-f, i, j were taken at X1000 magnification and scale bar **(j)** represents 20μm. A representative negative control can be seen in Figure 3.13. Arrows point to immunonegative Sertoli cell nuclei and arrowheads point to immunopositive Sertoli cells.

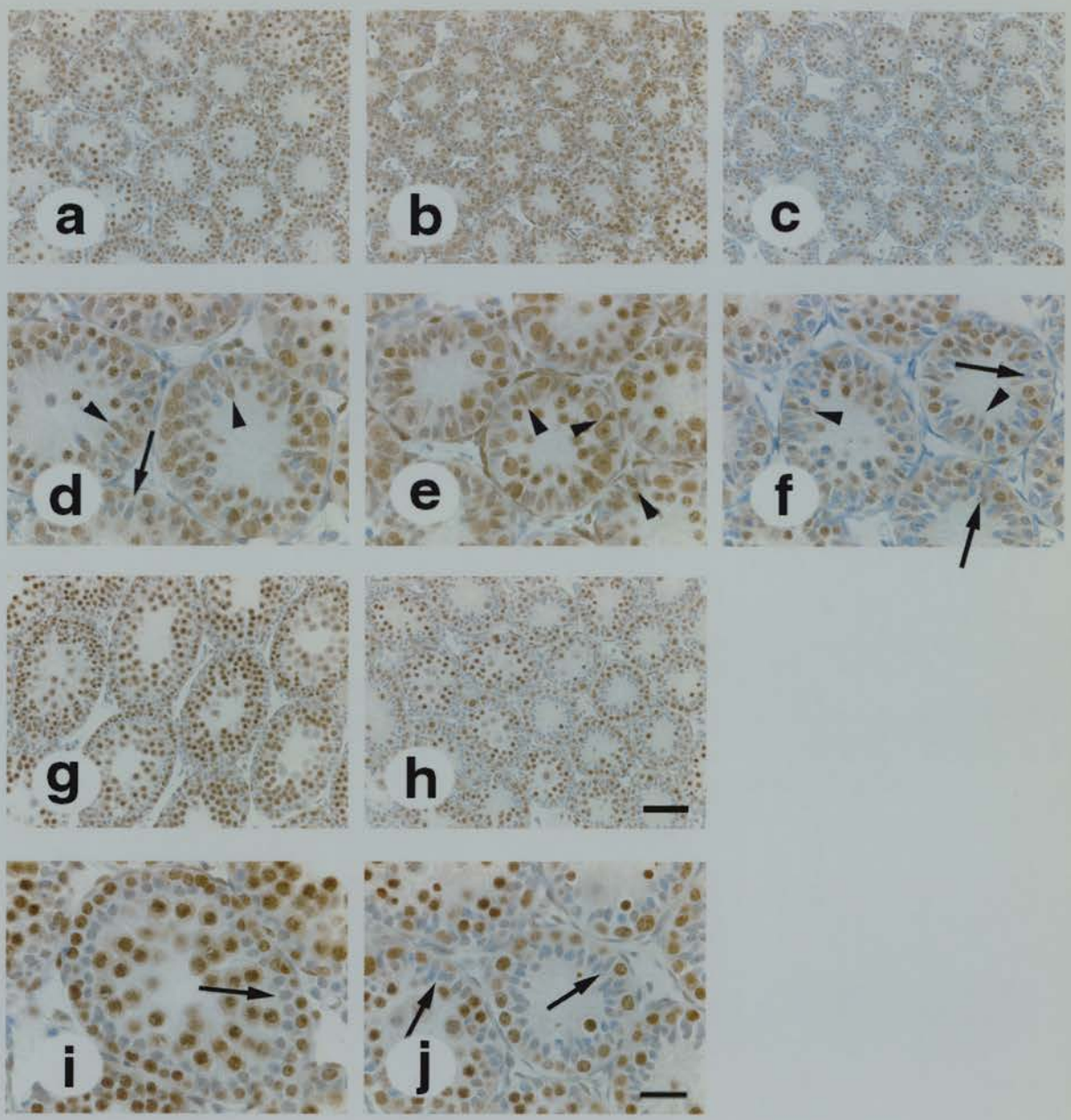

Figure 4.3: Immunolocalisation of PCNA to dividing cells in the postnatal rat testes following neonatal treatment with DES (10μg) and EE (10μg). Testes from rats aged postnatal day 18 **(a-f)** are shown following neonatal treatment with DES-10μg **(b, e)**, EE-10μg **(c, f)** and corn oil **(a, d)**. Testes from rats aged postnatal day 25 **(g-j)** are shown following neonatal treatment with DES-10μg **(h, j)** and corn oil **(g, i)**. Images a-c, g, h were taken at X400 magnification and scale bar **(h)** represents 50μm. Images d-f, i, j were taken at X1000 magnification and scale bar **(j)** represents 20μm. A representative negative control can be seen in Figure 3.2. Arrows point to immunonegative Sertoli cell nuclei. Arrowheads point to immunopositive Sertoli cell nuclei.

treated animals where the majority of Sertoli cell nuclei were immunonegative (Fig 4.3. a-f). In day 25 testes no immunopositive Sertoli cell nuclei could be seen in control or DES treated testes. However spermatogonia and early spermatocytes were strongly immunostained for PCNA at this age (Fig. 4. 3. g–j).

4.4. Discussion

The treatment regime used in the present study had been previously shown to cause a delay in Sertoli cell maturation and the onset of spermatogenesis in the postnatal rat testis. DES had a direct effect on the neonatal testis and modification of testis development was not solely due to suppression of gonadotrophin release from the anterior pituitary (Sharpe *et al.*, 1998). Testes from day 18 rats were used in the present study as previous results (Chapter 3) and published reports, confirmed that under normal conditions the majority of Sertoli cells in male Wistar rats had ceased dividing at this age and differentiation had been initiated. The pattern of PCNA immunolocalisation in the control day 18 testes in the present study was similar to the pattern of PCNA immunoexpression seen in previous studies (Chapter 3) demonstrating that the rats used in this study followed the normal pattern of rat testicular development.

It has been previously stated that SGP-1 is a marker of Sertoli cell chronological age and that expression of SGP-1 is not altered by the maturational state of the Sertoli cell (Bunick *et al.*, 1994). SGP-1 immunoexpression was not investigated in the present study as Sharpe et al., (1998) had previously shown a delay in the establishment of the normal pattern of SGP-1 immunoexpression following neonatal DES treatment consistent with the proposal that SGP-1 immunoexpression is regulated by Sertoli cell maturation.

Careful examination of the expression patterns of Musashi-1 and GATA-1 in the Sertoli cells of treated rat testes compared with controls suggested that neonatal

DES administration caused a slightly greater delay in testicular development than EE treatment. Previous results had shown an increase in Musashi-1 nuclear localisation with increasing Sertoli cell differentiation (Maguire *et al.*, 1999), Chapter 3). In the present study Musashi-1 immunoexpression in Sertoli cell nuclei of EE treated rats was more intense than that seen in nuclei of in DES-treated rat Sertoli cells. The difference in the extent of maturational delay between the two treatment groups was more marked when the pattern of GATA-1 immunoexpression was examined. A small number of day 18 testes from EE treated animals contained Sertoli cells with very low levels of GATA-1 expression. In contrast all Sertoli cell nuclei in DES-treated testes were immunonegative for GATA-1. These differences in the extent of maturational delay between the two treatment groups suggests that DES has a greater adverse affect on Sertoli cell maturation via ERβ than EE. One possibility is that the effect reflects differences in affinity for ERβ between the two oestrogens (Kuiper *et al.*, 1997).

In the present study delays in testicular development and Sertoli cell differentiation were demonstrated by the absence of stage specific expression of inhibin-α protein in both groups of treated rat testes recovered on day 18. Similar results were presented in Sharpe et al., (1998). The normal pattern of Musashi-1 immunoexpression characteristic of a mature Sertoli cell was delayed in both treatment groups: however the delay in Sertoli cell differentiation was more apparent in DES treated rat testes. No difference in GATA-4 immunoexpression between control and treated rat testes was observed. This was no surprise as immunolocalisation of GATA-4 protein remains fairly constant throughout normal Sertoli cell proliferation and differentiation. GATA-4 immunoexpression was included in the present study in an attempt to determine if GATA-4 expression was upregulated by DES administration as this had been previously observed in developing granulosa cells of three week old female rats (Heikinheimo *et al.*, 1997). GATA-4 expression was also shown to be upregulated at the mRNA level following FSH stimulation of MSC-1 Sertoli cells transfected with the FSH receptor (Heikinheimo *et al.*, 1997). No reduction in

GATA-4 expression was observed in the present study and this may be due to the fact that subtle changes in GATA-4 protein levels could not be detected using immunocytochemistry or that FSH levels were not sufficiently suppressed in the day 18 treated testis. Sharpe et al., (1998) stated that FSH levels in DES treated rats were not significantly reduced until day 35. The effect of oestrogens on GATA-4 expression in granulosa cells may be indirect. Oestrogen stimulates granulosa cell differentiation. Therefore the observed increase in GATA-4 expression following DES treatment may be an indirect effect whereby GATA-4 expression is upregulated coincident with granulosa cell differentiation. In addition ovarian GATA-4 expression was rapidly decreased following ovulation and the onset of granulosa cell apoptosis (Heikinheimo *et al.*, 1997). It is possible that GATA-4 functions as a cell proliferation and differentiation transcription factor in granulosa and Sertoli cells in the same way that GATA-4 expression is essential to cardiomyocyte differentiation and survival (Grepin *et al.*, 1995).

Comparisons of GATA-1 immunoexpression between neonatal day 18 treated and control testes showed a small decrease in immunoexpression following EE treatment. However GATA-1 protein expression was totally absent in day 18 DES treated testes. During normal testis development GATA-1 is first localised to Sertoli cell nuclei in a small population of Sertoli cells on day 14 of postnatal life and a day later immunoexpression is increased such that by day 18 the majority of Sertoli cells have strong nuclear immunolocalisation of GATA-1. This pattern of expression suggests that GATA-1 is only expressed in non-proliferating Sertoli cells undergoing the process of differentiation. Therefore DES treatment may not directly inhibit GATA-1 expression but by delaying differentiation and prolonging Sertoli cell proliferation DES administration prevents/delays GATA-1 protein expression. In order to address this theory GATA-1 immunolocalisation was investigated in testes from rats which had undergone the same neonatal DES treatment regime but were killed on day 25 rather than day 18. GATA-1 immunoexpression was observed in Sertoli cells of day 25 treated testes and therefore the absence of GATA-1 expression in day 18 DES treated rats appears to be due to an indirect effect of DES treatment on

Sertoli cell differentiation. To ensure that detection of GATA-1 immunoexpression in day 25 DES treated testes was not due to loss of DES from the general circulation resulting from the length of time between DES treatment (day 12) and testes removal (day 25), PCNA was immunolocalised to both day 18 and day 25 testes. Immunolocalisation of PCNA to day 18 testes indicated that a greater number of Sertoli cells continued to proliferate in DES treated testes when compared with the number of immunopositive PCNA Sertoli cells in control and EE treated rats. However by day 25 no immunopositive Sertoli cells could be observed in either control or treated rat testes. Therefore Sertoli cell replication had ceased by day 25 and all Sertoli cells had begun the process of differentiation and this was consistent with detection of GATA-1 protein in the nuclei of Sertoli cells.

It is therefore proposed that GATA-1 is only expressed in differentiating, non-proliferating Sertoli cells and may function as a transcription factor to regulate genes involved in cell differentiation. Although GATA-1, GATA-4 and Musashi-1 protein expression did not appear to be regulated by oestrogens the potential that ligand-dependent protein:protein interactions occurred between GATA-1 and/or GATA-4 and ERβ was not investigated. Both GATA-1 and GATA-4 have been shown to transactivate the inhibin-α promoter in Leydig tumour cell lines (Feng *et al.*, 1998; Ketola *et al.*, 1999). In the present study apart from the delay in stage specific expression associated with the delay in Sertoli cell maturation no significant changes in levels of expression of inhibin-α were noted. Proliferating Sertoli cells synthesise oestrogen during postnatal development and it has been suggested that oestrogen has a positive autocrine effect on Sertoli cell proliferation thus blocking Sertoli cell differentiation (Dorrington and Khan, 1993). Under normal conditions this autoregulatory loop is broken by paracrine and endocrine interactions which shut down Sertoli cell aromatase expression and therefore allow Sertoli cell differentiation to take place. It is possible that oestrogen bound to ERβ in the proliferating Sertoli cell interacts with GATA-1 which subsequently blocks the transactivation of genes essential for cell differentiation. Exogenous oestrogen administration may extend the period of

oestrogen/ERβ/GATA-1 interactions resulting in a delay in Sertoli cell differentiation. In order to investigate whether these protein:protein contacts do occur coimmunoprecipitation experiments between steroid receptors and GATA factors in Sertoli cells need to be carried out.

In conclusion inhibin-α, GATA-1 and Musashi-1 immunoexpression was modified with altered Sertoli cell maturation in the present study. GATA-1 and Musashi-1 protein expression was shown to be regulated by the stage of Sertoli cell maturation and consequently in the future both factors could be used as markers of Sertoli cell differentiation. In order to assess the direct effect(s) of steroid treatment on transcriptional regulation of Sertoli cell genes investigations employing isolated Sertoli cells were carried out (Chapter 5).

Chapter 5.

Effects of steroid treatment on isolated rat Sertoli cell gene expression

5.1. Introduction

Sertoli cell maturation takes place in a very dynamic and changing testicular environment. Differentiation of adult type Leydig cells occurs alongside Sertoli cell maturation and the onset of spermatogenesis (for review (Gondos and Berndston, 1994). All of these cellular changes contribute to the establishment of complex paracrine interactions within the postnatal testis. In parallel with Sertoli cell maturation, changes in the regulation of Sertoli cell function are influenced by a decline in FSH control concomitant with an increase in Sertoli cell androgen responsiveness (for review (Gondos and Berndston, 1994). Differentiated Sertoli cell function in vivo is regulated and influenced by numerous endocrine and paracrine interactions occurring during prepubertal testis development which can overshadow direct regulatory effects of steroids on Sertoli cell function when investigated in vivo. In previous chapters, expression patterns and regulation of known (inhibin-α, GATA-4 and GATA-1) and novel (Musashi-1) Sertoli cell proteins were investigated. The effect of neonatal E treatment on Sertoli cell protein expression was determined in vivo. However difficulty was found when determining direct modification of Sertoli cell function by E as changes in protein expression patterns could have arisen indirectly via E action on Sertoli cell maturation. Consequently in the present study direct effects of steroids on Sertoli cell gene expression were investigated using an in vitro primary Sertoli cell culture system where the complex paracrine and endocrine interactions present within the postnatal testis were removed allowing controlled manipulation of the Sertoli cell environment to be carried out.

Reports in the literature and preliminary results in previous chapters have provided evidence of possible regulation of GATA-1, GATA-4 and Musashi-1 Sertoli cell expression by E and T. For example differentiating mouse granulosa cells show an upregulation in GATA-4 mRNA expression following in vivo DES administration (Heikinheimo *et al.*, 1997). However T was not found to be essential for Sertoli cell GATA-4 protein expression in vivo (Ketola *et al.*, 1999). Ligand bound ERα has been shown to interact with GATA-1 in erythroid cells (red blood cell precursors) (Blobel and Orkin, 1996; Blobel *et al.*, 1995). It has therefore been proposed that ligand bound ERβ could interact with GATA-1 and GATA-4 proteins in Sertoli cell nuclei. Such interactions would inhibit transactivation of GATA regulated genes expressed in differentiating Sertoli cells including GATA-1 and inhibin-α (Feng *et al.*, 1998; Heikinheimo *et al.*, 1997). Both GATA-1 and Musashi-1 proteins are expressed in stage specific patterns; GATA-1 expression in Sertoli cells is restricted to stages V-XIII (Chapter 3) when AR levels within the seminiferous tubules are at the highest (Bremner *et al.*, 1994; Sharpe, 1994). In comparison, Musashi-1 Sertoli cell nuclear immunolocalisation is of greatest intensity during stages XI-VI (Maguire *et al.*, 1999) when AR levels within seminiferous tubules are at the lowest (Bremner *et al.*, 1994; Sharpe, 1994).

Forskolin is an activator of adenyl cyclase and mimics the effect of FSH on Sertoli cells through an upregulation in cAMP levels (Bicsak *et al.*, 1987). Sertoli cell expression of both inhibin-α protein and GATA-4 mRNAs are upregulated in vitro by FSH administration (Bicsak *et al.*, 1987; Heikinheimo *et al.*, 1997). Forskolin treatment of Sertoli cells was included in the present study as a control treatment to ensure that Sertoli cells in the in vitro system retained normal Sertoli cell characteristics and functions. Isolated Sertoli cells secrete inhibin B protein into culture media. Inhibin B secretion by Sertoli cells is modified by steroid treatment (Depuydt *et al.*, 1999). Therefore levels of inhibin B protein in Sertoli cell conditioned media were measured using an immunoassay to determine if the treatment regimes used in the present study modified the behaviour of the Sertoli cells in the culture system employed.

In the present study it was hoped to establish if oestrogen and testosterone, acting via ERβ and AR had a direct effect on isolated Sertoli cell gene expression of GATA-1, GATA-4, Musashi-1 and inhibin-α. Steroid regulation of Sertoli cell function was investigated at both mRNA and protein expression levels.

5.2. Materials and Methods

5.2.1. Rat primary Sertoli cell culture

5.2.1.1. Isolation of rat Sertoli cells

Testes were quickly removed from male Wistar rats, decapsulated, weighed and chopped into approximately 3mm pieces. Sertoli cells were isolated by enzymatic digestions using a modified method from Toebosch et al., (1988). Tubules were separated from the testis by gentle agitation in Hanks balanced salt solution (HBSS), (Gibco-BRL, Paisley, UK) with 0.25% trypsin (Gibco-BRL) and 13.5μg/ml deoxyribonuclease 1 (DNase 1) (Sigma) at 37°C for 25 minutes. At the end of the incubation 1.7mg of trypsin inhibitor (Sigma)/2g original tissue was added for 2 minutes at 21°C. Dispersed tubules were allowed to settle by unit gravity, washed in fresh HBSS and interstitial cells were removed in the supernatant. Separation of peritubular myoid cells from the seminiferous tubule walls occurred during incubation in 0.1mg/ml of collagenase type 1A (Sigma), 6.75μg/ml DNase 1 in HBSS at 37°C for 15 minutes. Contaminating germ cells were removed from Sertoli cell aggregates by repeated washing and centrifuging at 500g for 2 minutes. Washing and centrifuging was repeated until the supernatant was clear indicating that all germ cells had been removed in the supernatant. Clumps of Sertoli cells were plated out in Ham's F12 nutrient mixture with L-glutamine and sodium bicarbonate (Gibco-BRL), buffered with 1.5M Hepes pH 7.45, 50 Units/ml penicillin/streptomycin (Gibco-BRL) and 2.5μg/ml amphotericin B (Sigma) and were placed in a 32°C incubator at 5% CO_2 and 95% humidity.

Additional contaminating germ cells were removed after 24 hours, using hypotonic treatment as described by Galdieri et al., (1981). Briefly, medium was aspirated and 20mM Tris HCl, pH 7.4 was added to the cells at 21°C for 2.5 minutes. Fresh medium was added to the remaining Sertoli cells. Medium was replaced every 24 hours until cells and conditioned media were harvested after 5 days (120 hours) in culture.

5.2.1.2. Treatments

100μg of testosterone (Sigma, androsten-17β-ol-3-one) (T) was dissolved in 1ml absolute ethanol producing a stock solution at 100μg/ml and appropriate volumes were added to cell culture media to give a final working solution of 100ng/ml. A stock solution of oestradiol benzoate (Sigma) (E) in absolute ethanol (10μg/ml) was added to cell culture media such that a final working concentration of 10ng/ml was produced. Equivalent volumes of absolute ethanol were added to control media. Forskolin (Sigma) was dissolved in DMSO to give a stock solution at 10mM. Appropriate volumes were added to media to give a working concentration at 10μM.

All Sertoli cells were harvested at the same time after 5 days in culture. 5 day treatment of Sertoli cells began at time 0 hours when Sertoli cell cultures were set up and treatment continued until time 120 hours (5 days) when Sertoli cells were harvested. 24 hour treatment of Sertoli cells began at time 96 hours i.e. 24 hours before Sertoli cells were harvested and 4 hour Sertoli cell treatments were carried out at time 116 hours (4 hours before Sertoli cells were harvested). Therefore all Sertoli cells were in culture for the same amount of time.

5.2.2. RNA extraction and separation

RNA was extracted from whole rat tissue as described in Chapter 2.

5.2.2.1. Lysis of cultured cells

Sertoli cells cultured for RNA extraction were grown on 150mm and 100mm round culture dishes (Gibco). At time 120 hours, medim was carefully aspirated from the cells and any remaining medium washed away with PBS. Cells were

lysed in 750µl of Tri-Reagent™ and detached from the culture plate with a cell scraper, the solution was split between two 1.5 ml eppendorfs. An additional 250µl Tri-Reagent™ was added to the flask and used to remove any remaining cells.

5.2.2.2. RNA extraction

Sertoli cell RNA was extracted from lysed Sertoli cells in Tri-Reagent™ as described in Chapter 2 with appropriate adjustment of volumes.

5.2.2.3. RNA separation

10µg of total RNA extracted from cultured rat Sertoli cells and whole rat tissues was separated on 1.5% denaturing agarose gels and blotted onto nitrocellulose membranes as described in Sections 2.3.3 and Section 2.9, respectively. RNA was extracted from Sertoli cells in each treatment group. Experiments containing the same treatment groups were duplicated and RNA from each experiment was separated alongside total RNA extracted from day 18 whole rat testes and rat kidney which were used as control RNA samples.

5.2.3. Northern blot analysis

Northern blot analysis of total RNA was carried out as described in Section 2.9 and cDNA probes for SGP-1, inhibin-α, Musashi-1, GATA-4 and 18S were acquired as described in Chapter 3 and were radiolabelled following the protocol given in Chapter 2. Quantification of radiolabelled signals was carried out as described in Section 2.9/Chapter 3.

5.2.4. Immunocytochemistry

Sertoli cells cultured for immunocytochemical analysis were grown on glass chamber slides containing one or four culture wells (Lab-Tek, Nunc, Gibco). The treatment of Sertoli cells was carried out as described in section 5.2.1.2. and different timed treatments were set up on the same slide as shown below (Figure 5.1.)

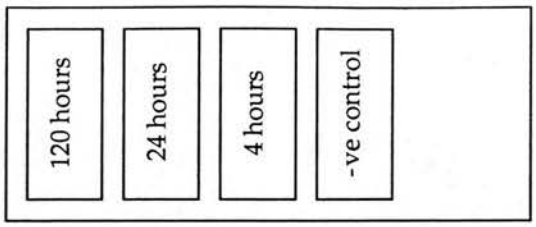

Figure 5.1: Diagram to show set up of timed treatment of isolated Sertoli cells cultured on 4 chamber glass slides. Isolated rat Sertoli cells were cultured in each chamber. At appropriate time points the media in each chamber was removed and replaced with media containing ethanol, F (10μM), E (10ng/ml) or T (100ng/ml).

At time 120 hours, medium was aspirated and cells were washed twice in PBS at 4°C for 5 minutes, slides were air dried and Sertoli cells were then fixed in 4% neutral buffered formaldehyde (NBF) for 10 minutes followed by two 10 minute washes in PBS at 4°C. Fixed Sertoli cells were stored in PBS at 4°C and immunocytochemical analysis was carried out within 48 hours of fixation.

After cultured Sertoli cells had been fixed onto the glass chamber slides all other slide fixtures attached to the glass slide were removed and the slides were air dried for approximately 10 minutes. Slides were briefly rehydrated in PBS prior to blocking of endogenous peroxidase for 20 minutes in PBS:H_2O_2 (3% v/v) followed by 2 x 5 minute PBS washes. Slides were permeabilised in PBS containing 10% v/v blocking serum (using normal serum from the animal that the secondary antibody was derived from), 1% w/v BSA and 0.1% v/v NP40 for 30 minutes. 2 x 5 minute PBS washes were carried out prior to application of 20% v/v blocking serum in PBS containing 5% w/v BSA. Blocking serum was removed after 30 minutes and primary antibody was applied at the appropriate dilution and left overnight in a humidified chamber at 4°C. Employment of secondary antibody and detection of immunolocalisation was carried out as described in Section 2.10 except that PBS was used in place of TBS.

5.2.4.1. Steroid receptors

Androgen receptor (AR). A rabbit polyclonal antibody (Santa Cruz) was used at a dilution of 1:100. In place of the biotinylated secondary antibody and avidin-conjugated horseradish peroxidase complex, DAKO EnVision + System (anti rabbit) with peroxidase (DAKO) was applied to the slides for 30 minutes, following manufacturers instructions. The system is based on an HRP labelled polymer which is conjugated to an anti-rabbit secondary antibody resulting in a more sensitive detection system. DAB was then applied to the sections as in section 2.10.

Oestrogen receptor beta (ERβ). A sheep polyclonal antibody raised against a peptide within the hinge domain of human ERβ (Saunders *et al.*, 2000) was used at a dilution of 1:2000. Biotinylated rabbit anti-sheep immunoglobulins were employed as secondary antibody (Vector).

5.2.4.2. Sertoli cell proteins

SGP-1. A rabbit polyclonal antibody, kindly donated by Dr. Steven Sylvester (University of Washington, Seattle) was used to immunolocalise SGP-1 to cultured Sertoli cells. The antibody was used at a dilution of 1:500 and biotinylated swine anti-rabbit immunoglobulins were used as the secondary antibody (DAKO).

Musashi-1. An anti-mouse–Musashi-1 monoclonal antibody raised in the rat according to standard procedures using a GST-mouse-Musashi-1 fusion protein (Sakakibara *et al.*, 1996) was a gift to Dr. Philippa Saunders from Professor H. Okano (Osaka University, Japan) and was used at a dilution of 1:1000. Biotinylated rabbit anti-rat immunoglobulins (Vector, Peterborough, UK) were used as the secondary antibody.

GATA-4. A goat polyclonal anti-rat GATA-4 antibody (SantaCruz) was used at a dilution of 1:250. Biotinylated rabbit anti-goat immunoglobulins (DAKO) were utilised as secondary antibodies.

5.2.4.3. Other proteins

Smooth muscle actin-α. A mouse monoclonal antibody (Sigma) was used to immunolocalise smooth muscle actin-α to peritubular myoid cell cytoplasm. The antibody was used at a dilution of 1:500 and rabbit anti-mouse biotinylated IgGs were used as the secondary antibody (DAKO).

The specificity of the specific antibodies (except for ERβ) was checked by replacing each antibody with normal serum from the animal species that the primary antibody was raised in. The normal serum was diluted to ensure protein content was the same as in the diluted primary antibody. In the case of ERβ, the peptide to which the primary antibody was raised against was incubated in the presence of the primary antibody overnight at 4°C. The concentration of incubated peptide was ten times that of the antibody with regards to protein content. The pre-absorbed antibody was then diluted to the working concentration which corresponded to that of the primary antibody. None of the above controls resulted in any signal above background.

Sections were analysed using an Olympus Provis microscope and images were captured onto computer using either a Kodak DCS420 camera or a Kodak DCS330 camera and stored on the computer using the Adobe Photoshop 5.0 programme.

5.2.5. Inhibin B assay

Sertoli cells in culture secrete inhibin B into culture media and in vitro Sertoli cell expression of inhibin B is regulated by steroid treatment (Depuydt *et al.*, 1999). Inhibin B secretion by cultured Sertoli cells was measured in the present study to establish if the treatment regimes used were appropriate.

Media from cultured Sertoli cells were collected at time 120 hours into 15ml centrifuge tubes (Corning, New York, USA) and centrifuged at 1500g for 10 minutes at 4°C to pellet any cell debris present. The supernatant was retained and placed in clean 15 ml centrifuge tubes and stored at –20°C until the inhibin B

assay was carried out. The inhibin B assay was very kindly carried out by Mr. Ian Swanston, Mrs Fiona Pitt and Mr. Paul Hartely using the protocol described in Groome et al., (1996) (Groome *et al.*, 1996). In preliminary experiments levels of inhibin B in Sertoli cell conditioned media could be detected in neat media however in later experiments levels of inhibin B in the conditioned media were too low to be detected using the assay. The Sertoli cell conditioned media was therefore concentrated 10 fold using Centrex® UF centrifugal ultrafilters for proteins sized 30 kDa and above (Schleicher and Schuell, New Hampshire, USA) and inhibin B levels in the concentrated media were then assayed. Each Sertoli cell treatment per experiment was carried out in duplicate and measurement of inhibin B levels in each sample were assayed in duplicate, the mean of which was calculated and used as the inhibin B value for that sample.

5.3. Results

5.3.1. Maintenance of normal Sertoli cell function and morphology after 5 days in culture

The majority of cells isolated for in vitro cell culture were identified as Sertoli cells by immunolocalisation of SGP-1 to Sertoli cell cytoplasm after 5 days in culture at 32°C (Fig. 5.2.a, Fig. 5.3.a). Other cell types present in Sertoli cell cultures were immunopositive for smooth muscle actin-α and cellular morphology suggested that they were peritubular myoid cells (Fig. 5.2.b). No quantitative determinations of cell contamination in Sertoli cell cultures were carried out. Hypotonic osmotic shock of germ cells and subsequent changes of media ensured that by day 5 the majority of contaminating germ cells had been removed. This was confirmed morphologically by light microscopy. A very small number of peritubular myoid cells remained in Sertoli cell culture at day 5 and could easily be detected due to the different size and morphology of the myoid cells. After 5 days in culture at 32°C both AR and ERβ were immunolocalised to Sertoli cell nuclei in the absence of T and E (Fig 5.3. b, c).

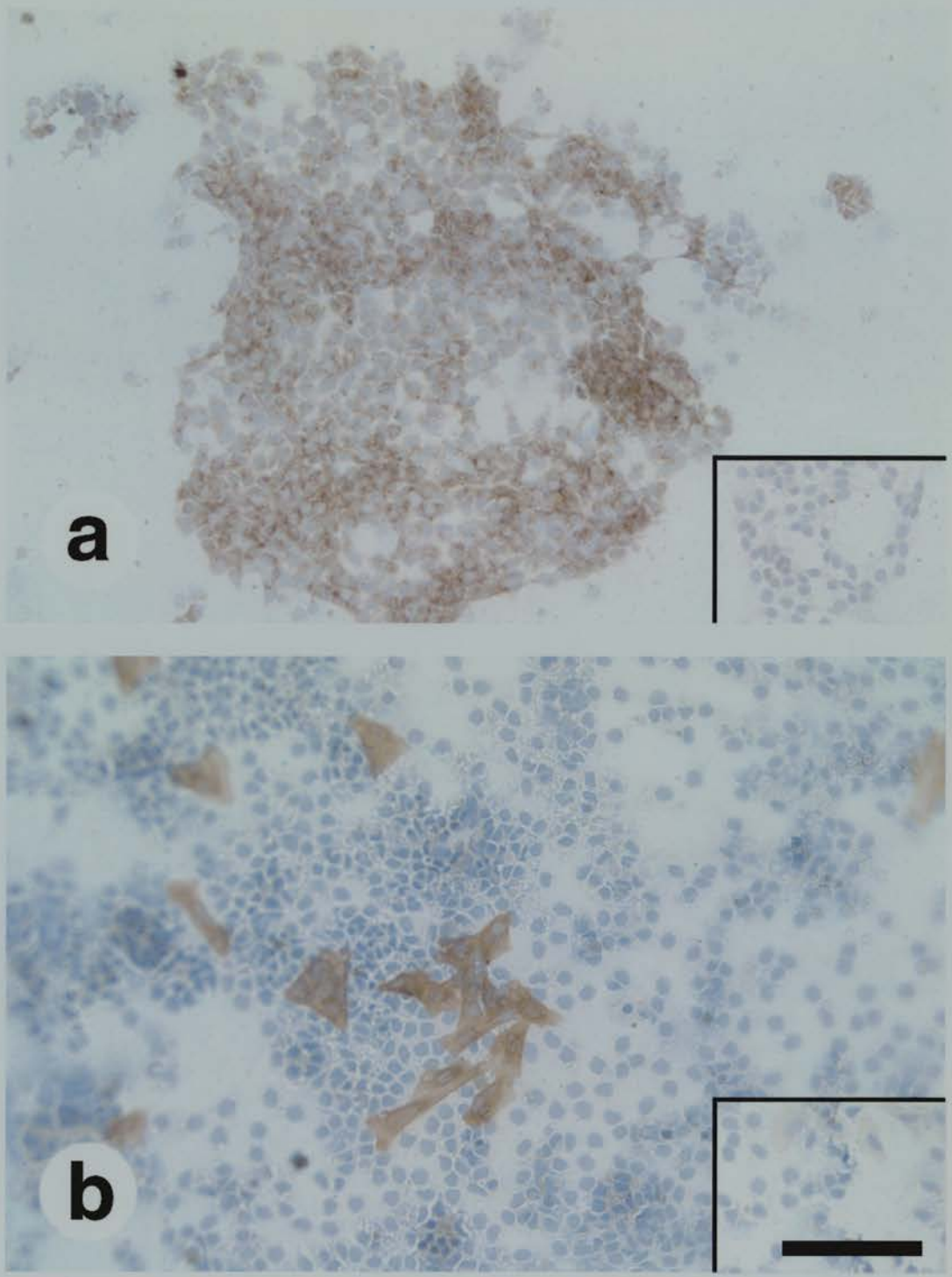

Figure 5.2: General appearance of isolated rat Sertoli cells in culture. SGP-1 was immunolocalised to the cytoplasm of Sertoli cells from postnatal day 18 rat testes which had been in culture for 5 days (120 hours) at 32°C **(a)**. Inset shows negative control when primary antibody was replaced with normal rabbit IgGs. Immunolocalisation of smooth muscle actin-α to the cytoplasm of peritubular myoid cells present in the Sertoli cell cultures **(b)**. Inset shows negative control when primary antibody was replaced with normal mouse IgGs. Scale bar represents 150µm.

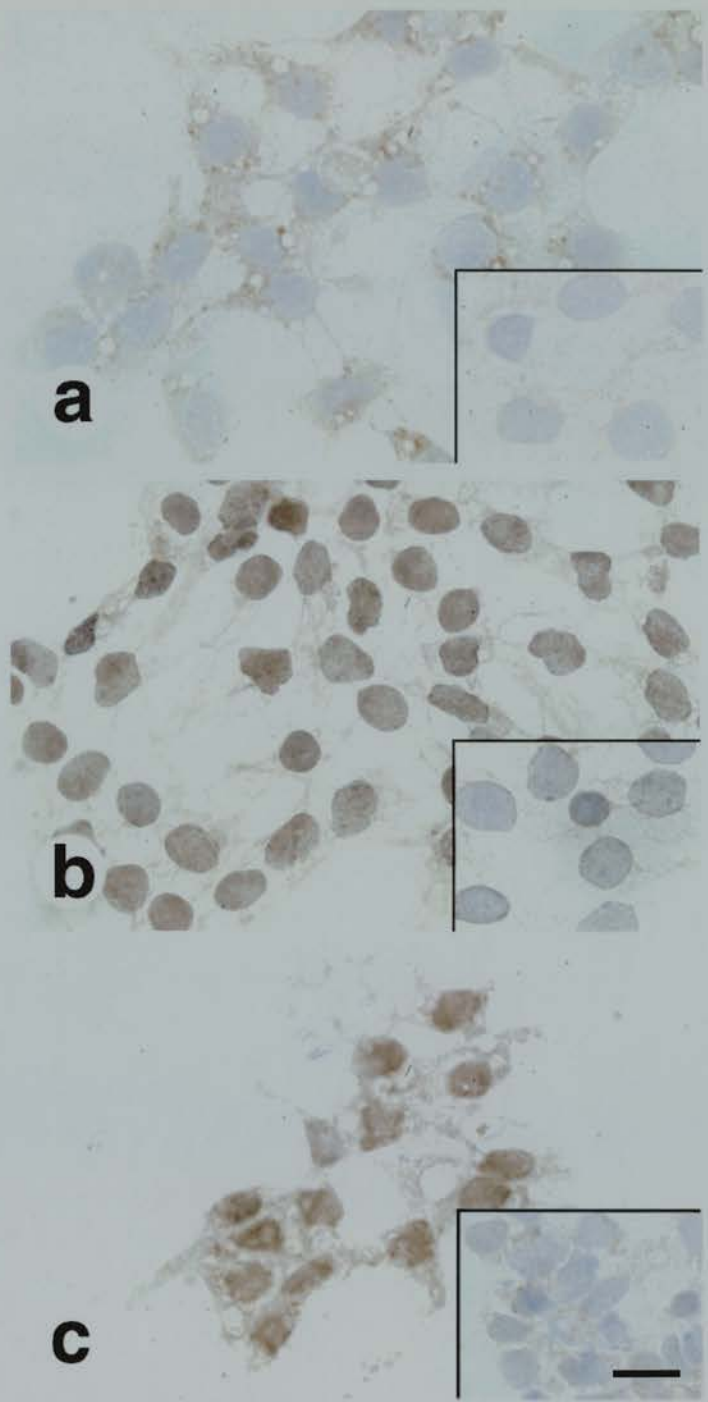

Figure 5.3: Immunoexpression of steroid receptors and SGP-1 to rat Sertoli cells in culture. Immunoexpression of SGP-1 to the cytoplasm of isolated rat Sertoli cells after 5 days (120 hours) in culture at 32°C **(a)**. Immunoexpression of androgen receptor (AR) **(b),** and oestrogen receptor β (ERβ) **(c),** in day 18 rat Sertoli cells cultured for 5 days (120 hours) in the absence of testosterone and oestrogen. Insets show negative controls when primary antibodies were replaced with normal rabbit IgGs **(a, b)** or preabsorbed peptide **(c)**. Scale bar represents 20μm.

5.3.2. Sertoli cell mRNA expression following steroid treatment

Northern blot analysis with an SGP-1 partial cDNA detected a single 2.6kb mRNA transcript in Sertoli cell RNA. No change in SGP-1 mRNA expression following F, E and T treatment for 24 and 4 hours could be observed on the Northern blot (Fig. 5.4.A, B). However following normalisation of SGP-1 mRNA expression with corresponding 18S rRNA levels from two separate experiments mean SGP-1:18S values were calculated and indicated that T treatment for 4 hours could upregulate SGP-1 mRNA expression in contrast to a possible downregulation in SGP-1 mRNA expression following 24 hour T administration (Fig. 5.4. C). E treatment did not appear to change SGP-1 mRNA levels whilst 24 hour F treatment resulted in a decrease in SGP-1 mRNA expression in this study.

A 1.5kb mRNA transcript was detected in Sertoli cell RNA using a radiolabelled inhibin-α partial cDNA. Inhibin-α mRNA expression was detected in day 18 rat testis and F 24 hour treated Sertoli cell RNA but inhibin-α mRNA expression in all other Sertoli cell RNA samples was too low to be detected using Northern blot analysis (Fig. 5.5. A). SGP-1 mRNA expression and 18S rRNA levels were determined on the same Northern blot and confirmed that Sertoli cell RNA was present in the lanes in which inhibin-α mRNA expression could not be detected (Fig. 5.5. B, C).

A 3kb Musashi-1 mRNA transcript was detected in Sertoli cell RNA samples using a Musashi-1 cDNA probe (Fig 5.6. A). Musashi-1 mRNA levels were low in E and ethanol (control) 4 hour treated RNA samples. Musashi-1 mRNA expression was undetectable in the T 24 hour treated Sertoli cell RNA sample. However Musashi-1 mRNA was detected in E, F and control 24 hour and T 4 hour treated RNA samples where SGP-1 and 18S Northern blot analysis showed increased amounts of total RNA had been loaded into these lanes (Fig. 5.6. B,

C). In a separate duplicate experiment (not shown) levels of Musashi-1 mRNA in all Sertoli cell samples were very low. Quantification of hybridisation signals was not carried out due to the absence or very low level of Musashi-1 hybridisation signals in a number of Sertoli cell RNA samples across both experiments.

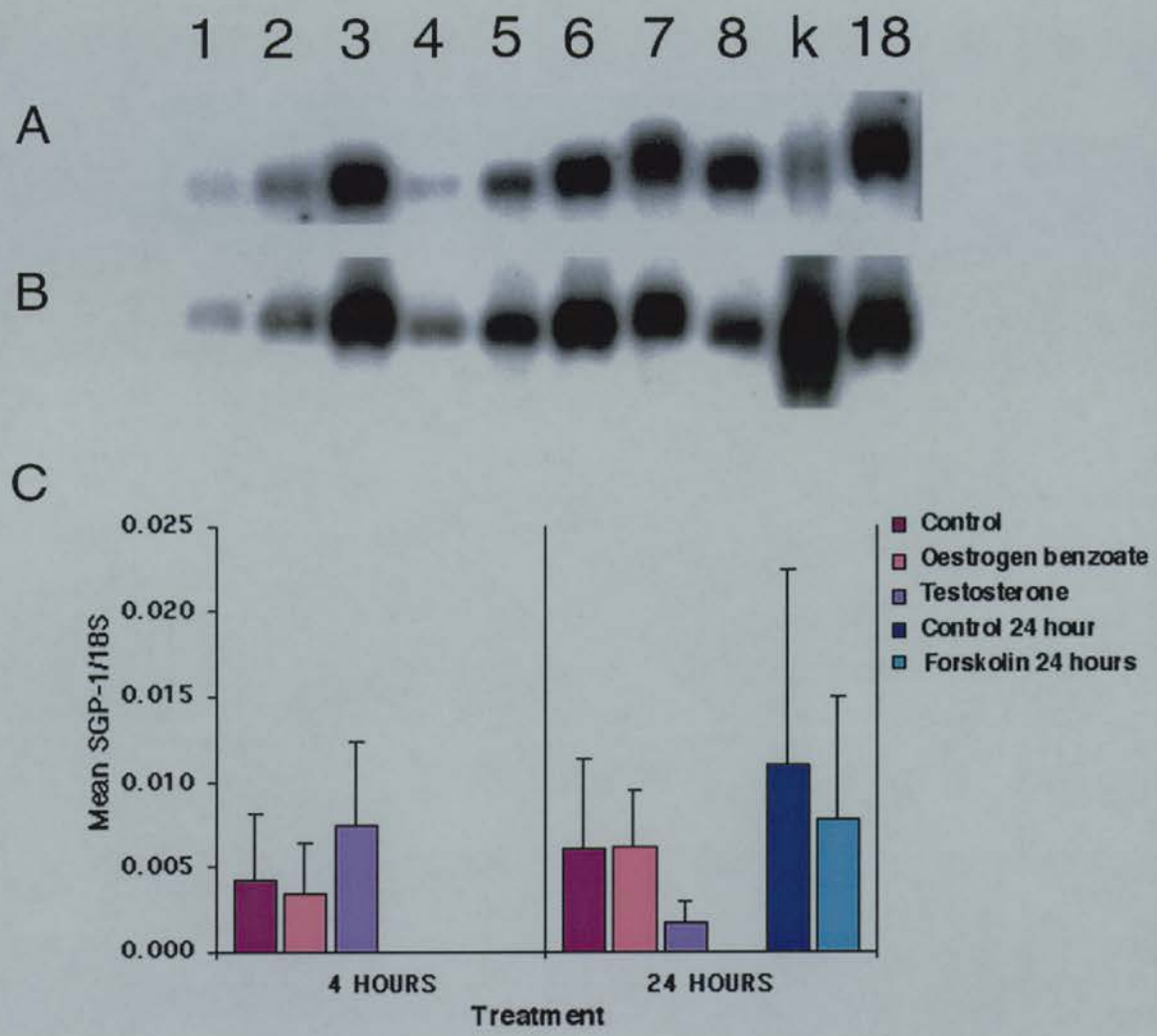

Figure 5.4: Northern blot analysis of SGP-1 mRNA expression in isolated rat Sertoli cell cultures following steroid treatment. All lanes were loaded with 10µg of total RNA extracted from day 18 rat Sertoli cells in culture following treatment with oestradiol benzoate (10ng/ml) for 4 hours **(1)** and 24 hours **(2)**; testosterone (100ng/ml) for 4 hours **(3)** and 24 hours **(4)**; ethanol for 4 hours **(5)** and 24 hours **(6)**. From a separate experiment Sertoli cells were treated with 10µM forskolin **(7)** and ethanol **(8)** for 24 hours. Total RNA extracted from rat kidney **(k)** and postnatal day 18 rat testis **(18)** were analysed as control RNA samples. RNA was hybridised with a ^{32}P-labelled SGP-1 cDNA which detected a 2.6kb transcript **(A)**. The RNA was reprobed with a ^{32}P-labelled 18S cDNA which hybridised to a 1.9kb 18S rRNA transcript and was used to check for even loading of RNA **(B)**. Following quantification of hybridisation signals the ratios of SGP-1 mRNA expression to 18S rRNA from two separate experiments was calculated and the mean SGP-1:18S ratios were plotted for each Sertoli cell RNA sample showing changes in Sertoli cell SGP-1 mRNA expression following steroid treatment **(C)**.

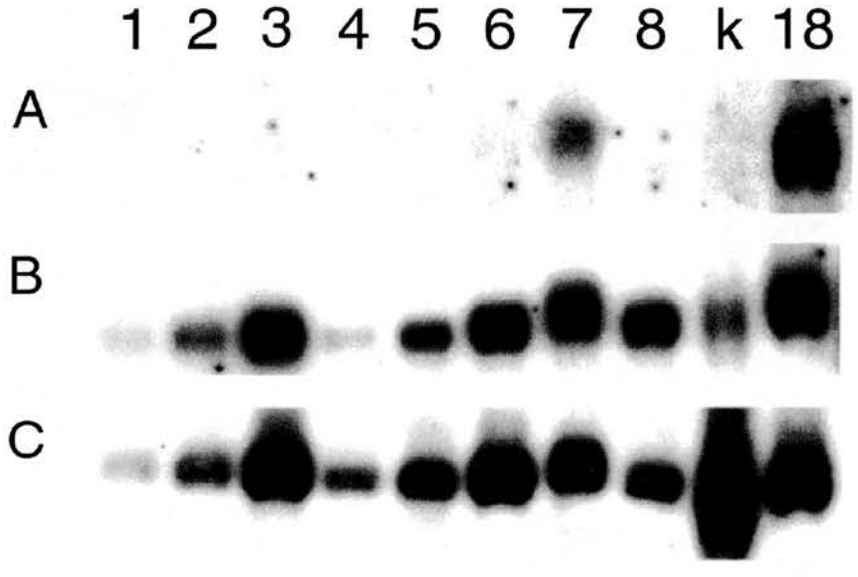

Figure 5.5: Northern blot analysis of inhibin-α mRNA expression in isolated rat Sertoli cell cultures following steroid treatment. All lanes were loaded with 10μg of total RNA extracted from day 18 rat Sertoli cells in culture following treatment with oestradiol benzoate (10ng/ml) for 4 hours **(1)** and 24 hours **(2)**; testosterone (100ng/ml) for 4 hours **(3)** and 24 hours **(4)**; ethanol for 4 hours **(5)** and 24 hours **(6)**. From a separate experiment Sertoli cells were treated with 10μM forskolin **(7)** and ethanol **(8)** for 24 hours. Total RNA extracted from rat kidney **(k)** and postnatal day 18 rat testis **(18)** were analysed as control RNA samples. RNA was hybridised with a ^{32}P-labelled inhibin-α cDNA which detected a 1.5kb transcript **(A)** in sample 7 and 18. RNA was reprobed with a ^{32}P-labelled SGP-1 cDNA which detected a 2.6kb transcript in all RNA samples **(B)**. RNA was then reprobed with a ^{32}P-labelled 18S cDNA which hybridised to a 1.9kb 18S ribosomal RNA transcript in all samples **(C)**.

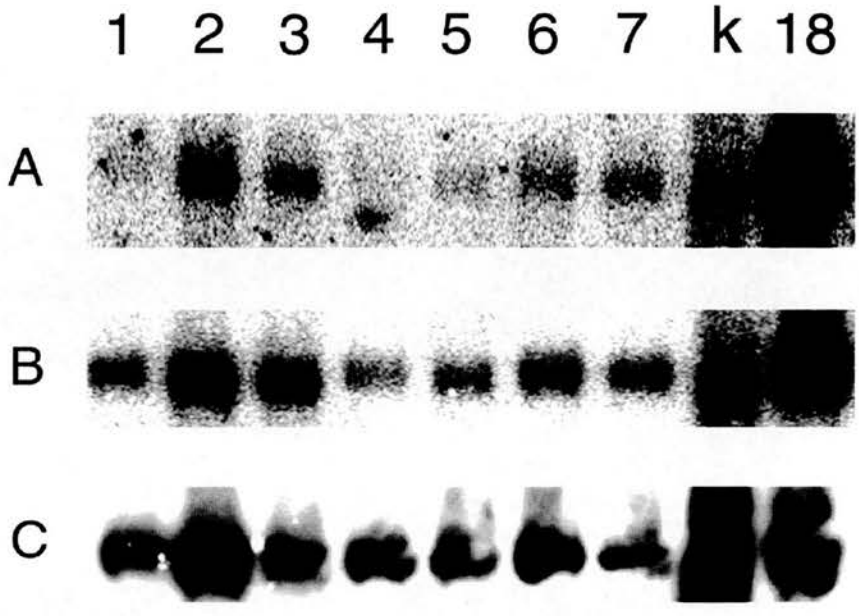

Figure 5.6: Northern blot analysis of Musashi-1 mRNA expression in isolated rat Sertoli cell cultures following steroid treatment. All lanes were loaded with 10μg of total RNA extracted from day 18 rat Sertoli cells following treatment with oestradiol benzoate (10ng/ml) for 4 hours **(1)** and 24 hours **(2)**; testosterone (100ng/ml) for 4 hours **(3)** and 24 hours **(4)**; ethanol for 4 hours **(5)** and 24 hours **(6)** and forskolin (10μM) for 24 hours **(7)**. Total RNA extracted from rat kidney **(k)** and postnatal day 18 rat testis **(18)**were analysed as control RNA samples. RNA was hybridised with a ^{32}P-labelled Musashi-1 cDNA which detected a 3kb transcript in all RNA samples **(A)**. RNA was reprobed with a ^{32}P-labelled SGP-1 cDNA which hydbrised to a 2.6 kb transcript in all RNA samples **(B)**. RNA was also reprobed with a ^{32}P-labelled 18S cDNA probe which detected a 1.9kb 18S rRNA transcript in all RNA samples **(C)**.

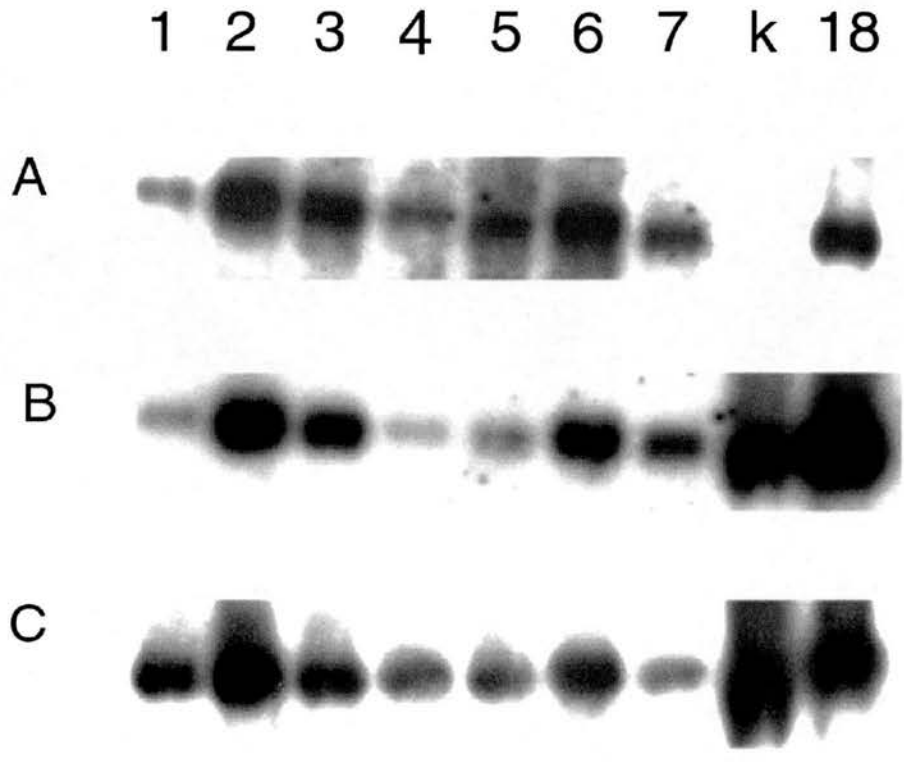

Figure 5.7: Northern blot analysis of GATA-4 mRNA expression in isolated rat Sertoli cell cultures following steroid treatment. All lanes were loaded with 10 μg of total RNA extracted from day 18 rat Sertoli cells in culture following treatment with oestradiol benzoate (10ng/ml) for 4 hours **(1)** and 24 hours **(2)**; testosterone (100ng/ml) for 4 hours **(3)** and 24 hours **(4)**; ethanol for 4 hours **(5)** and 24 hours **(6)** and forskolin (10μM) for 24 hours **(7)**. Total RNA extracted from rat kidney **(k)** and postnatal day 18 rat testis **(18)** were used as control RNA samples. RNA was hybridised with a ^{32}P-labelled GATA-4 cDNA which detected a 3.1kb RNA transcript **(A)**. RNA was reprobed with a ^{32}P-labelled SGP-1 cDNA which hybridised to a 2.6kb transcript **(B)** and a ^{32}P-labelled 18S cDNA was used to detect a 1.9kb 18S rRNA transcript **(C)**.

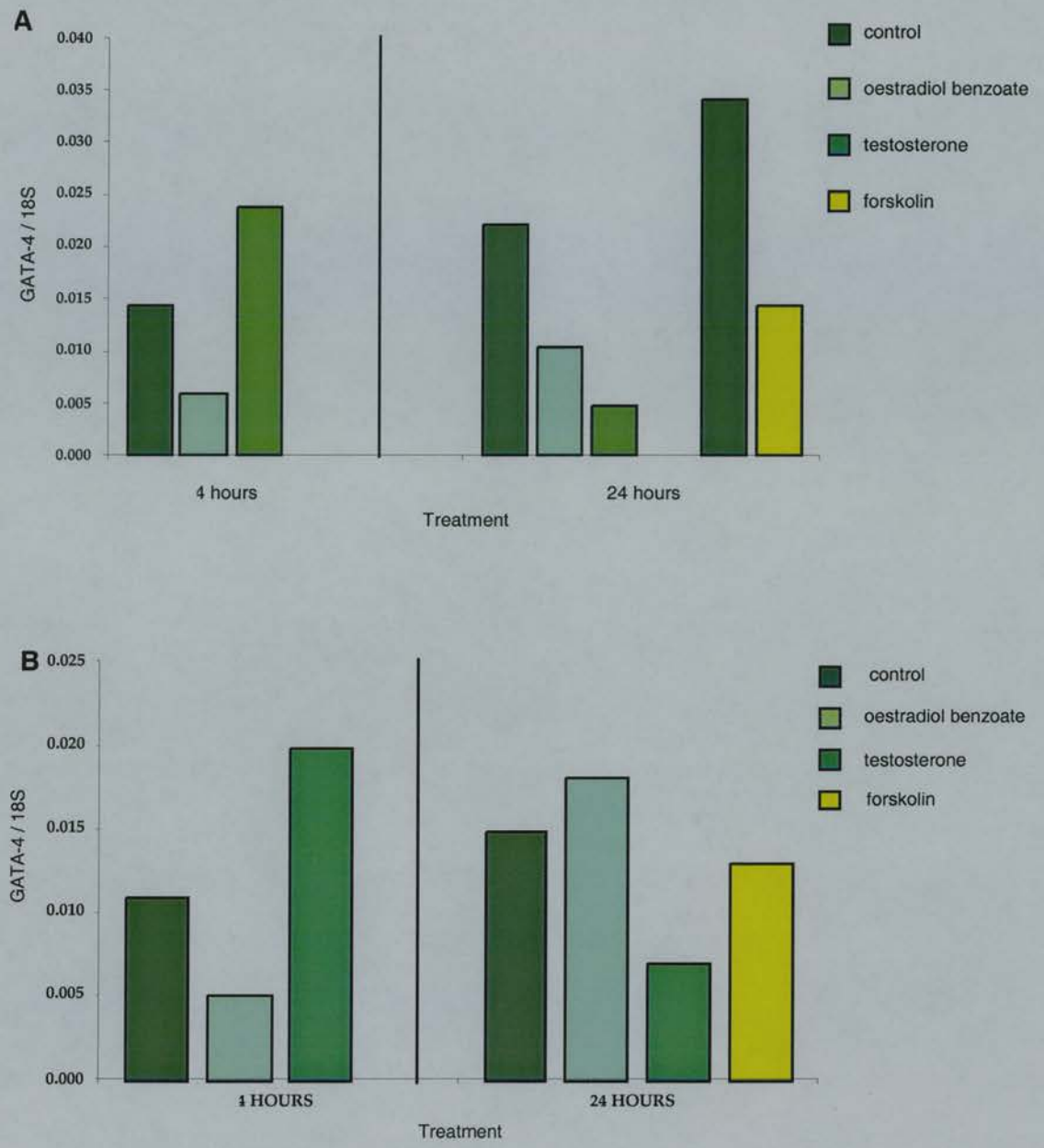

Figure 5.8: Changes in GATA-4 mRNA expression following steroid treatment of rat Sertoli cells in culture. GATA-4 mRNA expression was normalised against corresponding 18S rRNA values and the calculated GATA-4:18S results from two separate experiments are shown. In **(A)** forskolin (F) 24 hour treatment was carried out in a separate experiment and is shown alongside a corresponding 24 hour control sample from the same experiment.

A single 3.1kb GATA-4 mRNA transcript was detected in all Sertoli cell RNA samples using a radiolabelled GATA-4 partial cDNA (Fig. 5.7. A). GATA-4 hybridisation signals were quantified and normalised against corresponding 18S rRNA values. In Fig.5.8. both experiments (A and B) demonstrated a downregulation in rat Sertoli cell GATA-4 mRNA expression following E treatment for 4 hours and in contrast T treatment for the same time period upregulated GATA-4 gene expression. Following 24 hour treatment with T mRNA levels for GATA-4 were decreased in both experiments. However inconsistent results were obtained in both experiments following 24 hour E administration. This inconsistency in the results could be due to a non-specific mark (not shown) covering part of the GATA-4 signal in the Fig 5.8.B-24 hour sample. Subsequently the GATA-4 value for this sample will be artificially high. F treatment for 24 hours caused a downregulation in GATA-4 mRNA expression by Sertoli cells. However a greater decrease in mRNA levels was observed in experiment A.

5.3.3. Effect of steroid treatment on Sertoli cell immunoexpression

AR was immunolocalised to Sertoli cell nuclei after 5 days in culture in both the presence and absence of F, E and T (Fig. 5.9.). Some Sertoli cell nuclei were immunonegative for AR (black arrows) as were contaminating germ cells (blue arrowheads). Non-specific AR immunostaining in Sertoli cell cytoplasm could be observed (Fig. 5.9. h). Intensity of AR immunostaining in Sertoli cells treated with F (Fig. 5.9. b) and E (Fig. 5.9. c, f) was lower than the intensity of staining observed in control (Fig. 5.9. a, e) and T (Fig. 5.9. d, g) treated Sertoli cells.

ERβ was immunolocalised to Sertoli cell nuclei (Fig. 5.10.). A small population of Sertoli cells were immunonegative for ERβ (black arrows) and contaminating germ cells were also immunonegative (blue arrowheads). A small number of immunopositive peritubular myoid cell nuclei were observed and were identified due to their morphological appearance (Fig. 5.10. e). F treatment did not affect the intensity of ERβ immunostaining in Sertoli cell nuclei (Fig. 6.9. b, f,

h) when compared to control Sertoli cells (Fig. 5.10. a, e, i.). However following E and T treatment for 5 days (Fig. 5.10. c, d) the intensity of ERβ immunoexpression in Sertoli cell nuclei was slightly higher than in the nuclei of control Sertoli cell nuclei. Non-specific immunostaining was not observed (Fig. 5.10. inset).

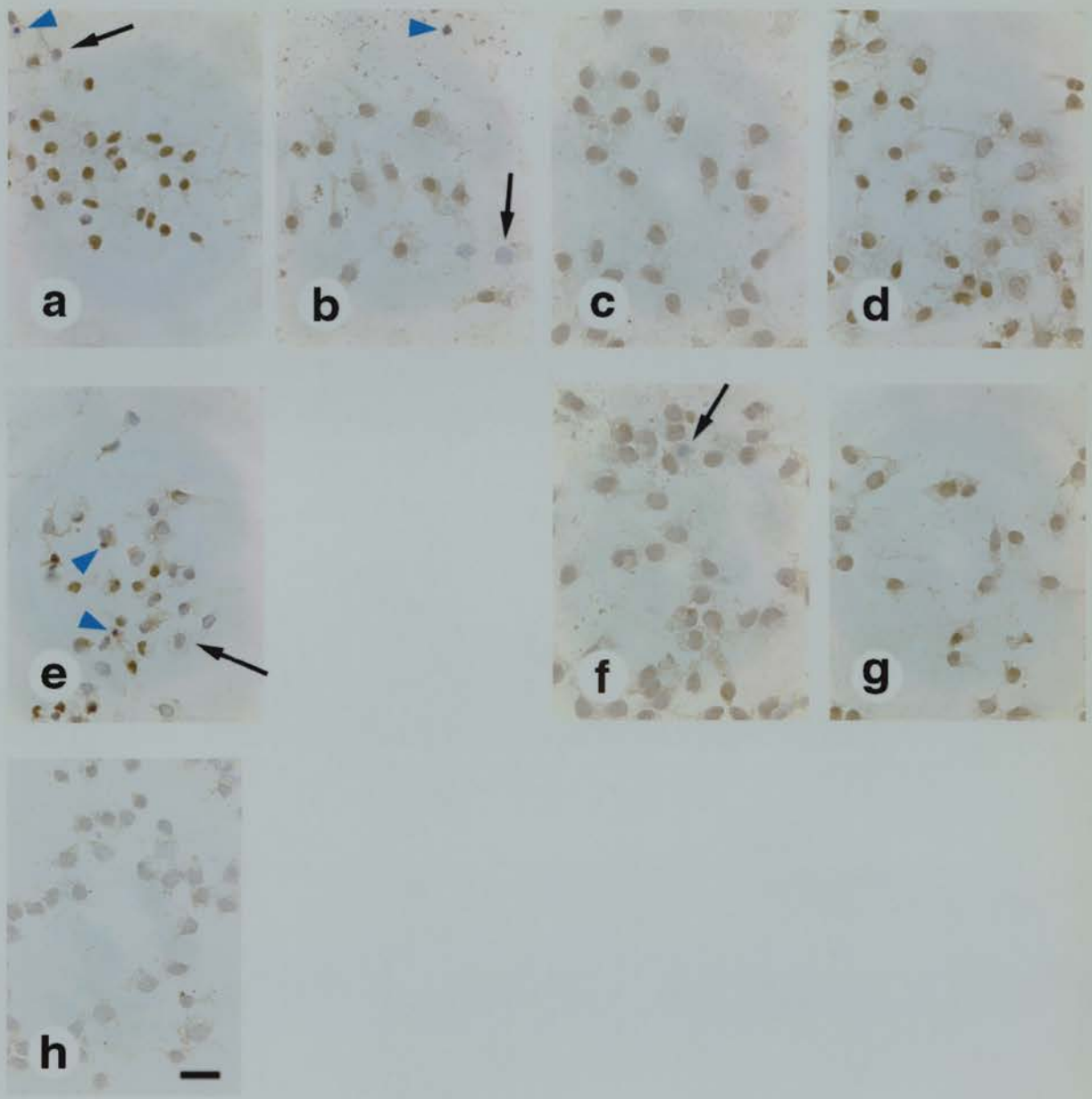

Figure 5.9: Effect of steroid treatment on AR immunolocalisation in cultured rat Sertoli cells. Sertoli cells were treated with ethanol **(a, e,)**, forskolin at 10μM **(b)**, oestradiol benzoate (E) at 10ng/ml **(c, f)** and testosterone (T) at 100ng/ml **(d, g)**. Treatments carried out for 5 days (120 hours) are shown in **a-d** and 24 hour treatments can be seen in **e-g**. A representative negative control is shown in **h** where primary antibody was replaced with normal rabbit IgGs. Black arrows point to immunonegative Sertoli cell nuclei and blue arrowheads point to contaminating germ cells. Scale bar represents 20μm.

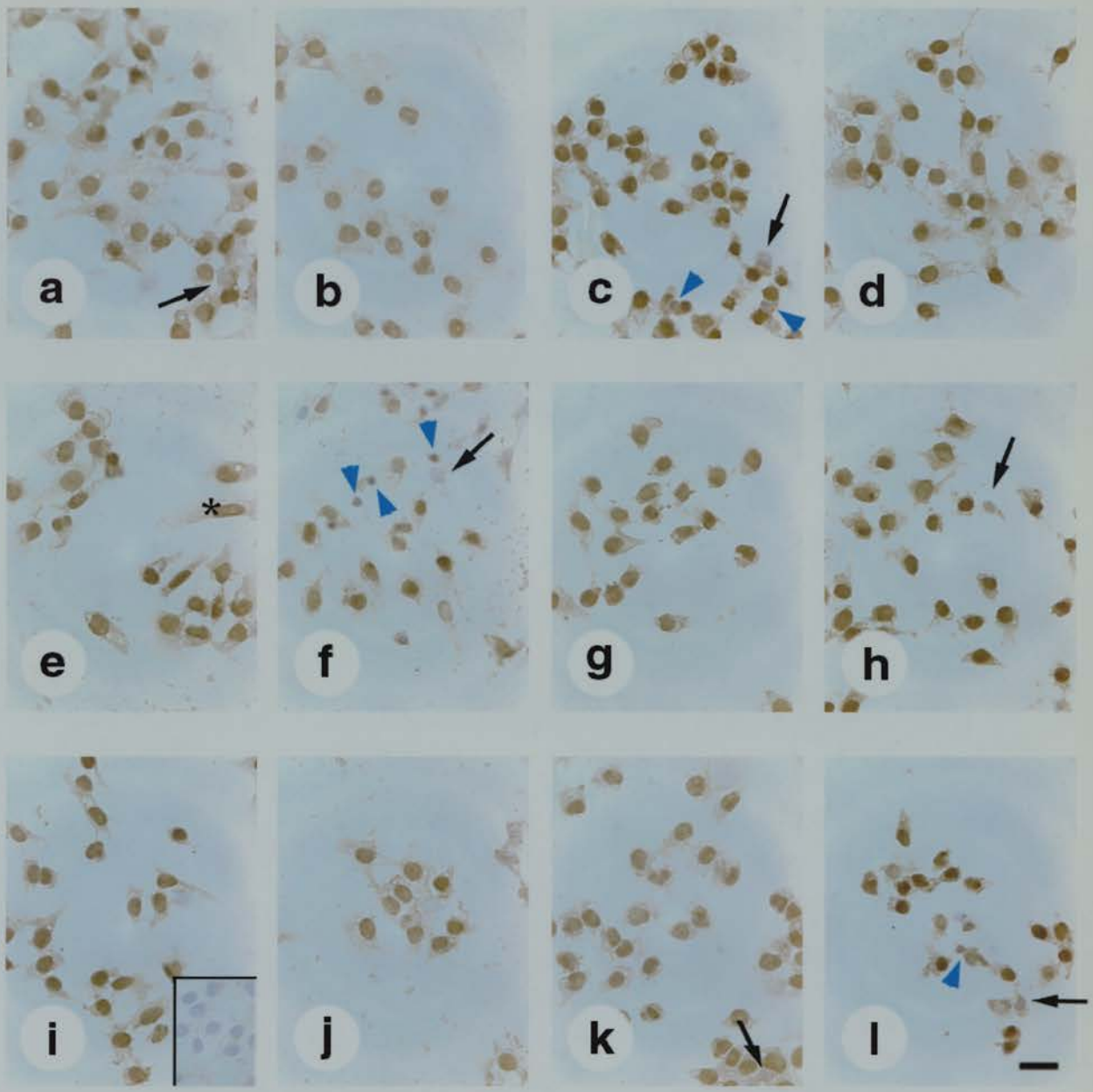

Figure 5.10: Effect of steroid treatment on immunolocalisation of ERβ to rat Sertoli cells in culture. Sertoli cells were treated with ethanol **(a, e, i)**, forskolin at 10μM**(b, f, j)**, oestradiol benzoate at 10ng/ml (E) **(c, g, k)** and testosterone at 100ng/ml (T) **(d, h, l)**. Treatments were carried out for 5 days (120 hours) **(a-d)**, 24 hours **(e-h)** and 4 hours **(i-l)**. Inset shows a representative negative control when primary antibody was replaced with preabsorbed peptide. Black arrows point to immunonegative Sertoli cell nuclei and blue arrowheads point to contaminating germ cells. Astericks marks immunopositive myoid cell. Scale bar represents 20μm.

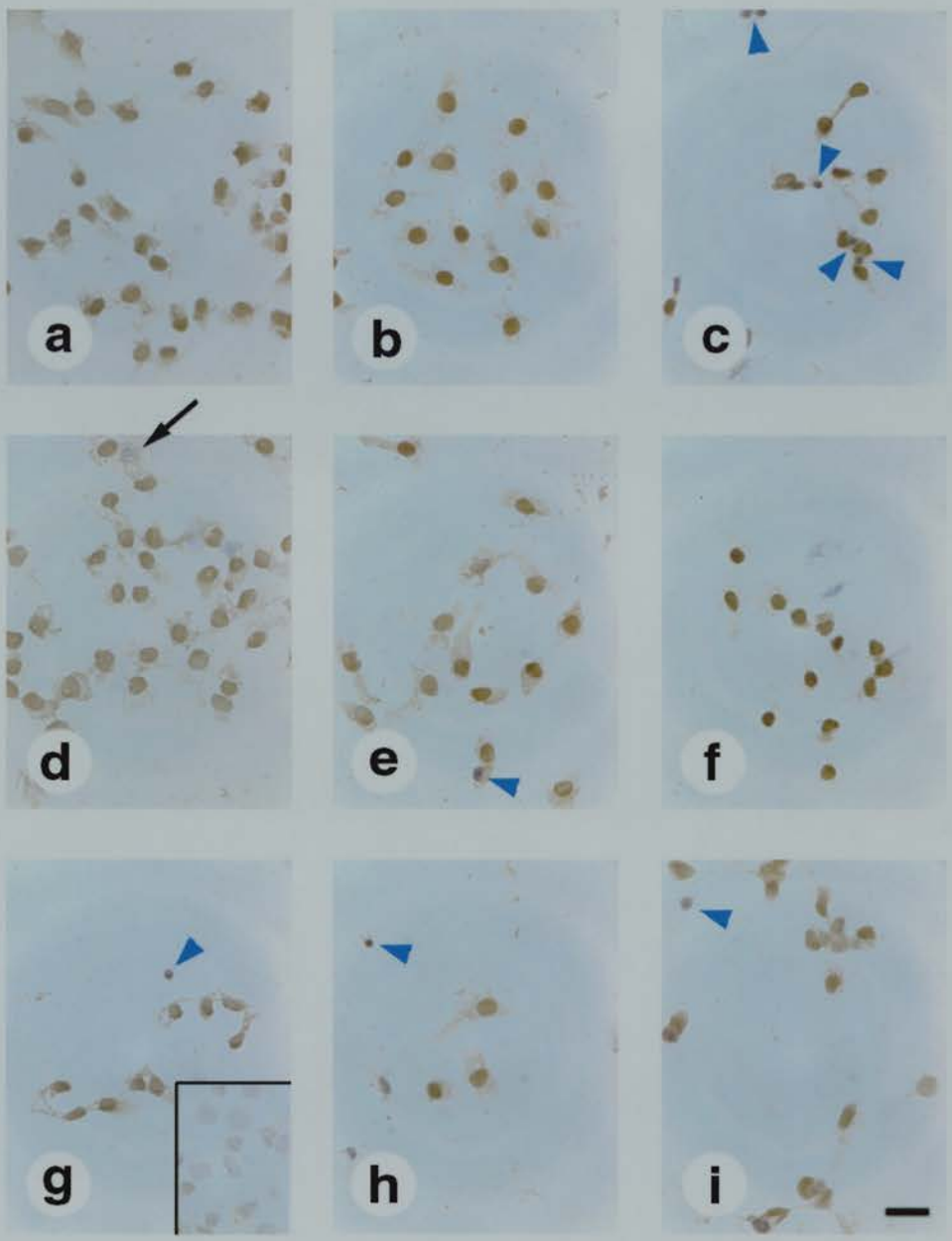

Figure 5.11: Effect of steroid treatment on immunolocalisation of Musashi-1 to rat Sertoli cells in culture. Sertoli cells were treated with ethanol **(a, d, g)**, forskolin at 10μM **(b, e, h)** and oestradiol benzoate at 10ng/ml (E) **(c, f, i)**. Treatments were carried out for 5 days (120 hours) **(a-c)**, 24 hours **(d-f)** and 4 hours **(g-i)**. Inset shows a representative negative control when primary antibody was replaced with normal rat IgGs. Black arrows point to immunonegative Sertoli cell nuclei and blue arrows point to contaminating germ cells. Scale bar represents 20μm.

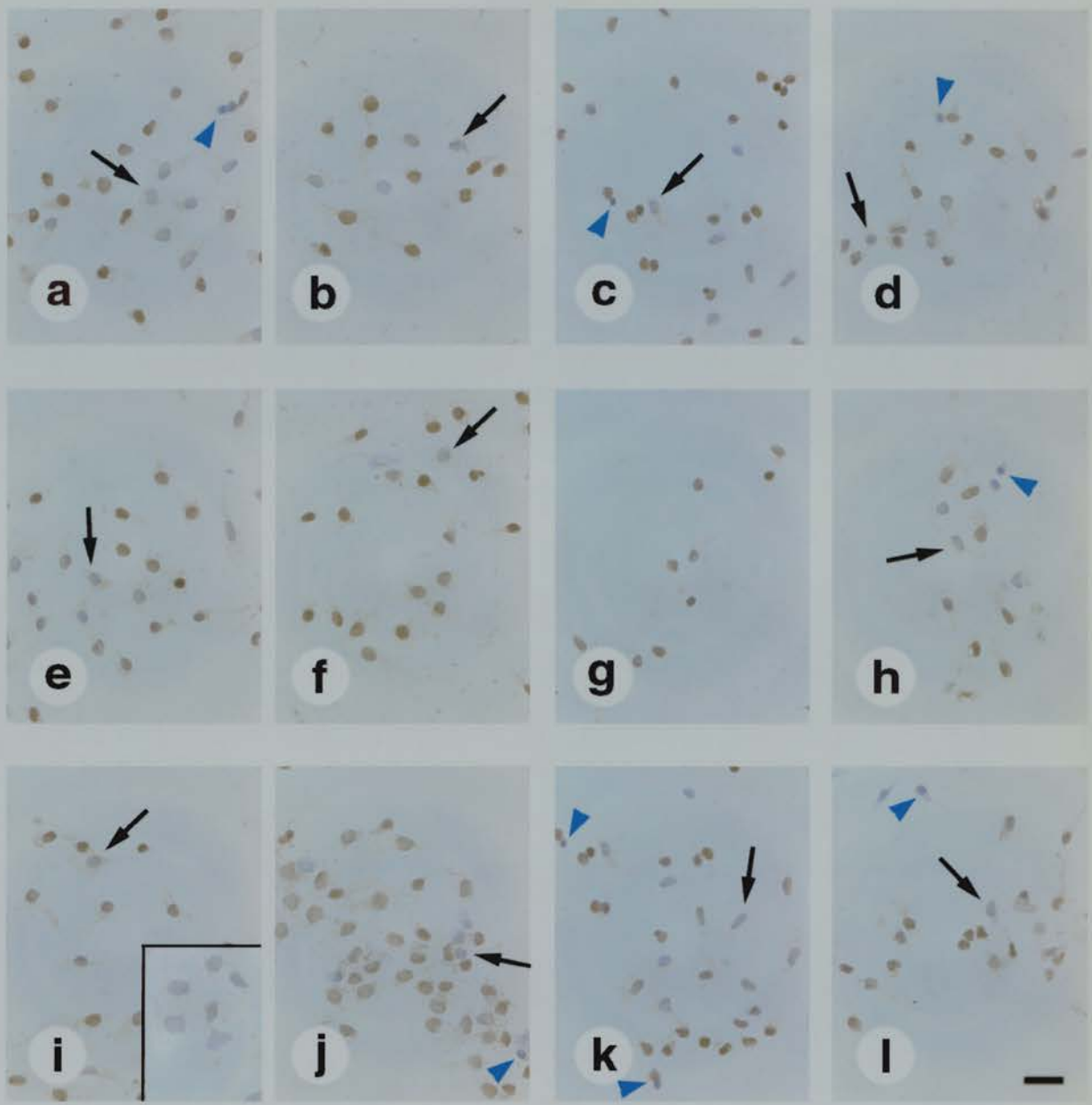

Figure 5.12: Effect of steroid treatment on immunolocalisation of GATA-4 to rat Sertoli cells in culture. Sertoli cells were treated with ethanol **(a, e, i)**, forskolin at 10μM**(b, f, j)**, oestradiol benzoate at 10ng/ml (E)**(c, g, k)** and testosterone at 100ng/ml (T) **(d, h, l)**. Treatments were carried out for 5 days (120 hours) **(a-d)**, 24 hours **(e-h)** and 4 hours **(i-l)**. Inset shows a representative negative control when primary antibody was replaced with normal goat IgGs. Black arrows point to immunonegative Sertoli cell nuclei and blue arrowheads point to contaminating germ cells. Scale bar represents 20μm.

Musashi-1 protein was immunolocalised to Sertoli cell nuclei (Fig. 5.11.). A very small population of Sertoli cells were immunonegative for Musashi-1 (black arrows) as were contaminating germ cells (blue arrowheads). Results for T treated Sertoli cells were only observed in one experiment. Musashi-1 protein expression did not appear to be changed with T treatment. However the effect of T treatment on Sertoli cells is not shown due to the fact that n=1. Following F and E treatment for 5 days, 24 hours and 4 hours, Musashi-1 immunostaining in Sertoli cell nuclei was of a greater intensity than in control Sertoli cell nuclei. The difference in intensity was most notable in E treated Sertoli cells (Fig. 5.11. c, f, i.). Non specific Musashi-1 immunostaining was not observed (Fig. 5.11. inset).

GATA-4 was immunolocalised to the nuclei of Sertoli cells (Fig. 5.12.). Some Sertoli cell nuclei were immunonegative for GATA-4 (black arrows) as were contaminating germ cells (blue arrowheads). No consistent change in the intensity of GATA-4 immunostaining could be observed with F, E or T treatment at any time studied. Non-specific GATA-4 immunostaining was not detected (Fig. 5.12. inset).

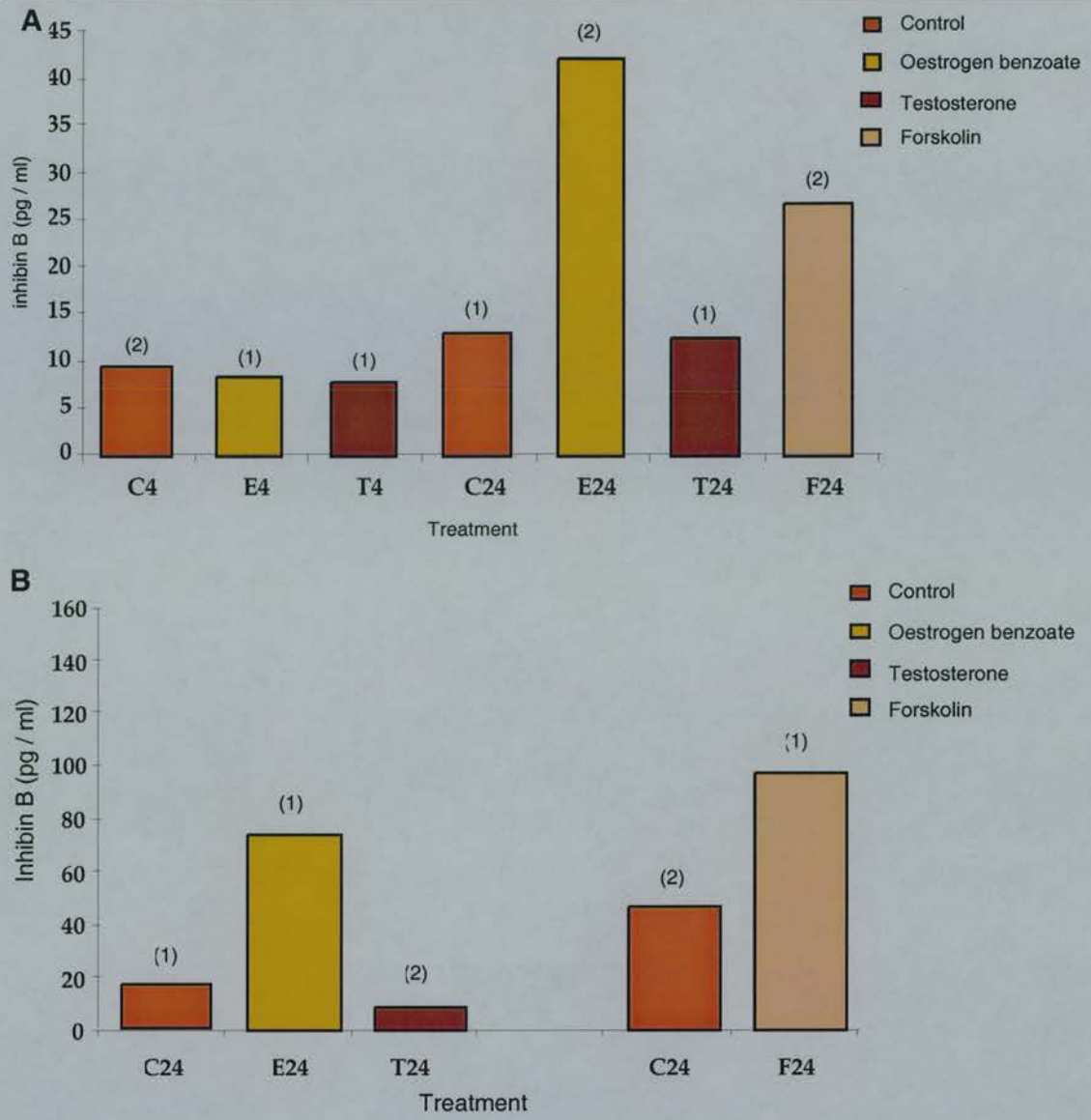

Figure 5.13: The effect of steroid treatment on secretion of inhibin B protein by rat Sertoli cells in culture. Day 18 rat Sertoli cells in culture were treated with ethanol (C), oestradiol benzoate (100ng/ml) (E), testosterone (10ng/ml) (T) and forskolin (10μM) (F) for 24 and 4 hours after which the conditioned media was removed and levels of inhibin B secreted into the media by the Sertoli cells were assayed. Each sample was assayed in duplicate and a mean of the duplicates was used as the sample inhibin B value. Numbers of separate samples assayed per treatment are given in brackets the mean of which is shown. In **(A)** where n=1 the level of inhibin B in the duplicate sample was undetectable by the assay whilst in **(B)** when n=1 the duplicate sample was not present. Inhibin B protein levels in the 4 hour treated samples in **(B)** were below the limits for detection in the assay.

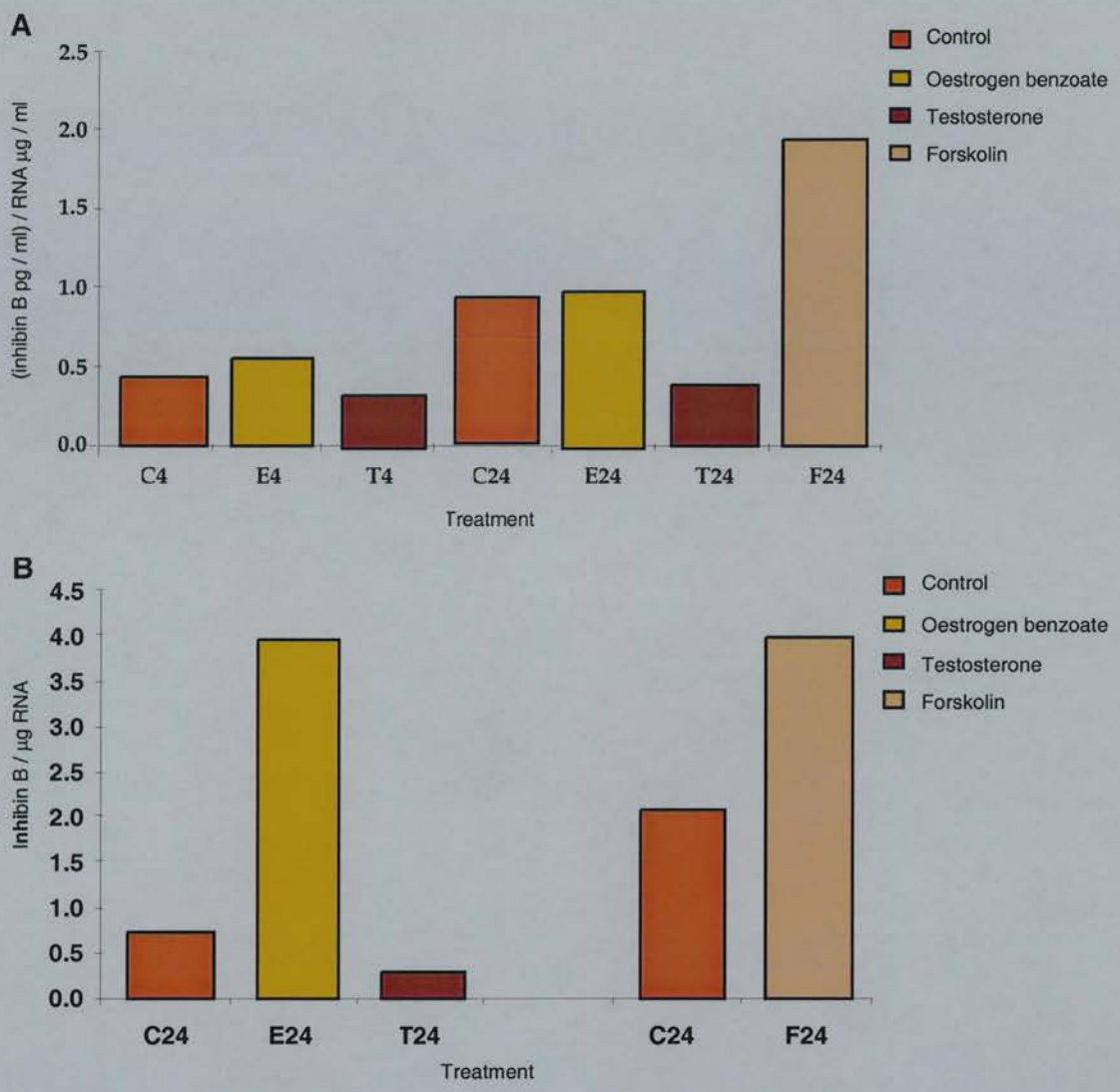

Figure 5.14: Changes in inhibin B secretion by cultured rat Sertoli cells following steroid treatment; inhibin B values have been normalised against the quantity of RNA obtained from each treatment group. Inhibin B values given in Fig. 5.13 were normalised against the amount of RNA obtained from each culture sample (as an indicator of Sertoli cell numbers). All results shown are n=1 as RNA was extracted from only one of the treated samples from each experimental group.

After inhibin B values had been normalised against the quantity of RNA extracted from corresponding Sertoli cell pellets, 24 hour F treatment upregulated inhibin B secretion in both experiments. E treatment also increased inhibin B levels in the media but not to the same extent as F (Fig. 5.13, 5.14). T

treatment on the other hand downregulated Sertoli cell inhibin B secretion. In both experiments 4 hour treated media contained less inhibin B protein than 24 hour treated media.

All the results shown in this chapter were from individual experiments which were duplicated and unless stated otherwise the results displayed are representative of the results obtained in the duplicate experiments.

5.4. Discussion

Sertoli cells from day 18 rats were employed in the present in vitro study as it has been previously shown that the majority of Sertoli cells in Wistar rats have ceased dividing by day 18 and were undergoing differentiation. Sertoli cells removed from testes of older animals would be further along the maturational process. However the mass expansion of germ cell populations accompanying Sertoli cell maturation was found to hinder the isolation and establishment of a 'pure' Sertoli cell primary culture (not shown). Therefore a balance was struck when obtaining primary cultures of Sertoli cells between the extent of Sertoli cell maturation and the expansion of germ cell numbers. Sertoli cells in the present study formed characteristic epithelial-like monolayers (Steinberger and Jakubowiak, 1993) after 3 days in culture and continued to express Sertoli cell specific proteins after 5 days in culture. SGP-1 protein was immunolocalised to Sertoli cell cytoplasm and expression of both AR and ERβ protein was maintained in the absence of E, T and F indicating that throughout the culture period Sertoli cells had not de-differentiated or lost normal cellular physiology. The inhibin B assay demonstrated that Sertoli cells had retained the ability to respond to hormonal treatment in the same way as reported in the literature (Anawalt *et al.*, 1996; Depuydt *et al.*, 1999; Turner *et al.*, 1989; Ultee-van Gessel *et al.*, 1986).

The changes in inhibin B levels also demonstrated that the administration of testosterone and oestradiol benzoate at the concentrations employed were

appropriate and capable of modifying Sertoli cell gene expression. 100ng/ml of testosterone had been previously shown in published data to modify rat Sertoli cell function and gene expression (Depuydt *et al.*, 1999; Foucault *et al.*, 1992; Lamb *et al.*, 1981; Lim *et al.*, 1994). Testosterone concentrations within the mature rat testis are thought to range from 25-100ng/ml (Sharpe, 1994). Therefore the concentration of testosterone used in the present study was within the normal physiological range found in the mature rat testis. Treatment of cultured rat Sertoli cells with E had not been studied extensively when this study was set up hence the administration of 10ng/ml of oestradiol benzoate (EB) had not been previously shown to modify Sertoli cell behaviour in vitro. The increase in inhibin B protein secretion in the presence of EB demonstrated that 10ng/ml of EB did alter Sertoli cell gene expression in vitro. Neonatal in vivo administration of EB to male rats has been previously shown to delay Sertoli cell maturation, cause dilation of the rete testis and backflow impairment of spermatogenesis (Aceitero *et al.*, 1998; Gaytan *et al.*, 1986) in addition to causing permanent atrophy of the testes and reduced serum FSH, LH and T levels (Bellido *et al.*, 1985; Bellido *et al.*, 1990; Brown-Grant *et al.*, 1975; Kincl *et al.*, 1965; Pinilla *et al.*, 1995; Tena-Sempere *et al.*, 2000). Similar abnormalities of the male reproductive tract in rats have been observed following neonatal administration of DES and EE (Fisher *et al.*, 1999; Fisher *et al.*, 1998; Sharpe *et al.*, 1998) demonstrating that oestrogenic chemicals can have a physiological effect on the rat testis. Forskolin treatment upregulated inhibin-α mRNA expression and increased GATA-4 and inhibin B protein production by isolated rat Sertoli cells which was in agreement with previously published literature (Bicsak *et al.*, 1987; Depuydt *et al.*, 1999; Heikinheimo *et al.*, 1997). It was not investigated whether the observed effects of F on Sertoli cell gene expression were due to the administration of F or the vehicle, DMSO. Stocco et al., (1995) observed an inhibition in StAR protein production in cultured MA-10 Leydig cells following administration of 5% DMSO. 0.1% DMSO was used in the present study and to the best of my knowledge there are no reports of DMSO at this concentration modifying the function of cells in vitro.

Sertoli cell inhibin B assay results were normalised against differences in numbers of Sertoli cells present at the time of media recovery by dividing inhibin B values by the total mass of RNA extracted from the corresponding cell pellet/culture dish. Inhibin B levels were down regulated in the presence of T and were markedly upregulated following F treatment as had been previously reported in the literature (Anawalt *et al.*, 1996; Bicsak *et al.*, 1987; Depuydt *et al.*, 1999; Keinan *et al.*, 1989; Toebosch *et al.*, 1989; Ultee-van Gessel *et al.*, 1986). However when protein production was normalised against approximate cell numbers the extent of inhibin B upregulation by E was greatly reduced in experiment A (see Figs. 5.13, 14). This observation highlighted the technical problem experienced in the current study of obtaining and maintaining equal numbers of Sertoli cells between treatment groups and experiments. Plating out of Sertoli cells was performed by pipetting equal volumes of Sertoli cell suspension onto the culture plates. Due to the presence of Sertoli cell aggregates and contaminating germ cells when Sertoli cells were first isolated, final numbers of Sertoli cells varied between treatment groups. Initially it was decided that the different numbers of Sertoli cells between treatment groups could be c normalised by correcting mRNA expression levels against SGP-1 mRNA values. SGP-1 is constitutively expressed by Sertoli cells (Chapter 3) and is often used in the literature as a control Sertoli cell gene by which expression of other Sertoli cell factors are standardised. In the present study SGP-1 mRNA expression levels were normalised against 18S rRNA values and SGP-1 expression in the cultured rat Sertoli cells was upregulated after 4 hour T administration. In contrast SGP-1 mRNA levels were downregulated following T treatment for 24 hours. Due to these novel findings, 18S rRNA values were used to normalise Sertoli cell mRNA expression and although this was not ideal due to the contribution of contaminating cells to 18S rRNA values, levels of contamination were considered to be too low to markedly alter results.

However it is well established that differences in cell density can dramatically affect Sertoli cell function and morphology and therefore simple correction using 18S rRNA values may not be sufficient. For example increasing the cell

density of day 20 rat Sertoli cells caused a decrease in E production (Schteingart *et al.*, 1995). In contrast levels of steroid receptor protein are increased in parallel with increased Sertoli cell density (Nakhla *et al.*, 1984). Therefore it is generally agreed that the density at which cells are cultured can influence cellular behaviour and physiology. Even though differences in mRNA mass and cell numbers were controlled in the present study modification of Sertoli cell function (e.g. protein expression and steroid receptor numbers) by varying cell densities could not be controlled and consequently changes in Sertoli cell gene expression observed cannot be definitely stated to result from hormonal regulation but maybe caused by differences in cell densities between treatment groups.

Immunocytochemical techniques were useful in determining the subcellular location of steroid receptors and other Sertoli cell protein expression in vitro. However the effects of hormonal treatment on levels of protein expression were not accurately quantified using immunocytochemistry. Due to time constraints further investigations using a range of antibody concentrations were not carried out which was unfortunate as subtle changes in intracellular protein levels may not have been detected because of the high concentrations of primary antibody that were employed. Sertoli cells adhered well to one chambered glass slides but cells did not adhere as well to smaller four chambered glass slides. This may be due to the increased surface tension of media in the smaller chambers which resulted in a greater number of Sertoli cells floating on the surface of the medium rather than sinking and adhering to the glass. Although smaller media volumes were employed to overcome this effect, Sertoli cell numbers in four-chambered slides remained low and subsequently the effect of short term hormonal treatment on AR protein expression and T treatment on Musashi-1 protein expression could not be determined due to inadequate numbers of cells in some culture chambers. Due to the smaller surface area in the four-chambered slides difficulty was also experienced in removal of contaminating germ cells which were usually dislodged from Sertoli cells by swirling of the media between media changes.

The 'swirling action' was reduced in the smaller chambers which resulted in inadequate removal of the germ cells. Thus the number of contaminating germ cells in Sertoli cell cultures grown on glass slides was greater than the number of germ cells remaining in Sertoli cell cultures grown on plastic dishes.

AR protein was expressed in cultured Sertoli cell nuclei in the absence and presence of F, EB and T. T is known to upregulate the level of AR protein expression in cultured Sertoli cells. In contrast T administration decreases or causes no detectable change in AR mRNA levels (Blok *et al.*, 1989; Cardone *et al.*, 1998; Sar *et al.*, 1993; Verhoeven and Caillaeu, 1988). It has been proposed that T administration increases the stability of AR protein structure and does not directly increase AR gene expression. T administration did not alter detectable AR protein levels in the present study. In day 18 rat testis Sertoli cells are becoming increasingly responsive to androgens at the expense of FSH regulation (Gondos and Berndston, 1994). Altered T levels had no effect on AR protein levels in day 21 male Sprague–Dawley rats (Shan *et al.*, 1994) and it is possible that in the day 18 male Wistar rats used in the present study the increase in androgen responsiveness is delayed in comparison to other strains of rat. It is also a possibility that an upregulation in AR protein levels following T administration was not observed due to the length of T treatment (5 days and 24 hours). Sanborn et al., (Sanborn *et al.*, 1977) demonstrated a 30% decrease in AR nuclear accumulation 1 hour following T administration. Therefore some ligand-AR complexes may have dissociated 24 hours after T administration. However if this was the case an increase in cytosolic AR levels might have been observed. It has been reported that E decreases AR levels in Sertoli cells at both mRNA and protein expression levels in vivo (Sharpe *et al.*, 1998; Tena-Sempere *et al.*, 2000) and in vitro (Cardone *et al.*, 1998; Collins *et al.*, 1994). A slight decrease in the intensity of AR immunostaining was observed in Sertoli cell nuclei following EB treatment for 5 days (120 hours) and 24 hours. A similar decrease in AR protein levels was observed following 120 hours of F administration. Previous research has shown that FSH treatment of cultured Sertoli cells upregulates AR levels (Blok *et al.*, 1989; Sanborn *et al.*, 1991;

Verhoeven and Caillaeu, 1988). FSH also increases Sertoli cell aromatase expression and therefore any endogenous T present in the cell culture system could be metabolised into E which could then decrease AR protein levels. In addition F is known to dephosphorylate AR protein in cultured LnCAP cells (Blok *et al.*, 1998). Dephosphorylation prevents ligand binding to the AR and consequently endogenous T would be prevented from binding to AR resulting in the observed downregulation in AR levels following F administration.

The intensity of nuclear ERβ Sertoli cell immunostaining was increased following EB and T administration. The intensity of ERβ immunostaining was greatest when Sertoli cells were constantly exposed to E and T for 120 hours. In vivo EB treatment caused a direct upregulation of ERβ mRNA levels in immature and prepubertal rat testis (Tena-Sempere *et al.*, 2000). However to my knowledge there are no previous reports of T upregulating ERβ protein levels in Sertoli cells. In neonatal rat cardiac myocytes T indirectly upregulates ERβ mRNA levels via aromatisation (Grohe *et al.*, 1998) and aromatisation of T could also occur in the present study. F treatment has been shown to downregulate ERβ mRNA levels in cultured granulosa cells (Byers *et al.*, 1997) and a similar effect of F on ERβ protein levels was also observed in the present study.

Steroid regulation of Sertoli cell gene expression was assessed at both mRNA and protein levels. RTPCR analysis of mRNA expression levels is a more sensitive technique than Northern blot analysis. However accurate quantification of mRNA levels was required in the present study and therefore Northern blot analysis was employed. Preliminary investigations had detected GATA-1 mRNA expression in the isolated rat Sertoli cells using RTPCR. However GATA-1 gene expression could not be detected in the cultured Sertoli cells using Northern blot analysis and immunocytochemistry. Onodera et al., (1997) reported that GATA-1 protein expression was lost in mouse Sertoli cell cultures between days 1 and 3, although RTPCR studies demonstrated GATA-1 mRNA was expressed in Sertoli cells cultured over five days it is possible that

GATA-1 expression levels were very low by this stage of culture and consequently could not be detected using the techniques employed in the current study. Inhibin-α protein could not be detected in the isolated rat Sertoli cells using immunocytochemistry and consistent with this inhibin-α mRNA was only detected in F treated Sertoli cells. Upregulation of inhibin protein levels following F administration in cultured rat Sertoli cells was previously demonstrated by Bicsak et al., (1987). It appears that Sertoli cells had not lost the ability to express inhibin-α during the culture period but basal levels were too low for detection using Northern blot analysis. In previous reports larger volumes of Sertoli cell RNA have been required for detection of inhibin-α mRNA in rat Sertoli cell cultures (Keinan *et al.*, 1989; Klaij *et al.*, 1990; le Magueresse-Battistoni *et al.*, 1995; Toebosch *et al.*, 1988). Expression of Musashi-1 in cultured rat Sertoli cells has not been previously reported. However levels of Musashi-1 mRNA were also at the limit of Northern blot detection such that in order to determine the effects of steroids on Musashi-1 and inhibin-α gene expression increased mass of RNA or RPA analysis will be needed in future studies. Musashi-1 protein was immunolocalised to Sertoli cell nuclei as well as to Sertoli cell cytoplasm but at a lower intensity than nuclear staining. Thus Sertoli cells in vitro express Musashi-1 protein in a similar pattern to the characteristic immunostaining observed in differentiating cells in vivo (Chapter 3). Following E and F treatment Musashi-1 protein levels were upregulated in Sertoli cell nuclei. However the possible significance of these findings is unknown and therefore requires further investigation.

GATA-4 gene expression was investigated in the present study in an attempt to determine if E had a direct affect on GATA-4 expression. EB treatment of Sertoli cells for 4 hours downregulated GATA-4 mRNA expression. (Upregulation of GATA-4 mRNA expression following E treatment for 24 hours observed in Fig. 5.8.B is artificially high due to a non specific mark on the Northern membrane covering part of the GATA-4 signal for this sample). Heikinheimo et al., (1997) showed that DES administration upregulated GATA-4 mRNA expression in cultured granulosa cells. However these differences in the effect of oestrogens

on GATA-4 expression may result from different regulatory systems existing in the different cell types. In addition as has been previously discussed in Chapter 4 oestrogens may indirectly increase GATA-4 mRNA levels in granulosa cells via increasing cellular differentiation. T treatment of Sertoli cells for 4 hours upregulated GATA-4 mRNA levels whilst 24 hour administration had the opposite effect on GATA-4 mRNA. FSH has been previously shown to significantly upregulate GATA-4 mRNA levels in MSC-1 Sertoli cells transfected with an FSH receptor gene and F significantly increased GATA-4 mRNA levels in cultured mouse granulosa cells (Heikinheimo *et al.*, 1997). In the present study F decreased GATA-4 mRNA levels in the cultured rat Sertoli cells. During Sertoli cell maturation FSH responsiveness of Sertoli cells begins to decline with a parallel increase in regulation of Sertoli cell function by T (Gondos and Berndston, 1994). It is therefore proposed that in day 18 rat Sertoli cells in culture the effect of F (FSH) on GATA-4 gene expression is declining whilst T regulation of expression is increasing and consequently F does not increase GATA-4 mRNA levels whilst T has a negative effect on GATA-4 gene expression. The relevance of these findings is uncertain due to the fact that GATA-4 protein levels remained unchanged following steroid treatment. However this could result from high antibody concentrations employed in the present study which could mask subtle changes in GATA-4 protein levels. Moreover in vivo levels of GATA-4 protein in Sertoli cells appear constant throughout development and across all spermatogenic stages.

In the present study expression of both AR and ERβ proteins in cultured Sertoli cells was maintained in the absence of either T or EB. The cultured Sertoli cells also retained the ability to respond to hormonal treatment in patterns characteristic of Sertoli cells in vivo. Preliminary data regarding steroid regulation of ERβ protein expression in cultured rat Sertoli cells was reported in addition to the regulation of GATA-4 mRNA and protein levels by EB and T. The expression of Musashi-1 mRNA and protein has not previously been reported in cultured rat Sertoli cells. However further investigations are

necessary in order to determine if steroid regulation of Musashi-1 takes place in the differentiating Sertoli cell.

In addition these preliminary investigations highlighted a number of problems that are associated with primary cultures of Sertoli cells. The importance of obtaining equal distribution of Sertoli cells across treatment groups was demonstrated as were the problems associated with achieving adequate numbers of cells to adhere to smaller chambers on glass slides. Insufficient cell numbers on slides not only restricts the amount of information that can be obtained but results may be confounded by problems associated with variations in cell density. Low cell numbers also restrict the quantities of RNA and protein that can be extracted from the cultures which in turn limits the analysis of mRNA levels to the more abundant mRNA transcripts. All these aforementioned problems are linked to the number of Sertoli cells harvested in the primary isolation procedure as day 18 rat Sertoli cells have a very low mitotic index and numbers do not appear to increase during the culture period. One option to try to overcome the above problems is to utilise an immortalised Sertoli cell line in order to address the direct actions of steroids on Sertoli cell gene.

Chapter 6.

Steroid treatment of an immortal mouse Sertoli cell line (SK11).

6.1. Introduction

Regulation of Sertoli cell behaviour has been studied using primary Sertoli cell cultures where the extracellular environment of the Sertoli cell can be more easily manipulated than in vivo and direct effects of steroids can therefore be investigated. However as was described in Chapter 5 Sertoli cells in primary culture begin to lose their normal functions including the ability to respond to hormones between day 6 and day 10 of cell culture (Steinberger and Jakubowiak, 1993). Low levels of germ cell and peritubular myoid cell contamination can also complicate interpretation of results due to the possible regulation of Sertoli cell behaviour by paracrine interactions with these cell types. In addition investigation of the regulation of gene expression in Sertoli cells is greatly hampered by the requirement for large numbers of Sertoli cells to obtain replicate determinants in primary culture systems. The number of Sertoli cells used in vitro is often limited by the number of cells obtained at the time of cell isolation. Such problems in cell culture can be overcome by utilising an immortalised Sertoli cell line where sufficient numbers of identical Sertoli cells can be acquired without the need to sacrifice additional animals.

A number of immortalised Sertoli cell lines have been generated by insertion of immortalising genes into primary cultured Sertoli cells. However normal cellular physiology can be altered in these cells (Ridley *et al.*, 1988). Jat et al., (Jat *et al.*, 1991) generated a transgenic mouse that had stably integrated the simian virus 40 (SV40) mutant temperature sensitive A58 (tsA58) large tumour antigen (TAg) gene which is a conditional immortalising gene under the control of the mouse major histocompatibility complex class 1 promoter (H-2k^b) which is active at various levels in different tissues of the body. The tsA58 TAg gene product is functional at the permissive temperature of 33°C

but is rapidly degraded at the non-permissive temperature of 39°C. Thus the presence of the TAg gene results in the generation of continuously proliferating cell lines which are capable of differentiation following inactivation of the tsA58 TAg gene when placed at 39°C.

A number of immortalised cell lines have been obtained from H-2K^b-tsA58 transgenic mouse tissues. The cells have been reimplanted into mouse tissue after in vitro culture and have subsequently differentiated and functioned normally in the intact organism (Chambers *et al.*, 1993; Morgan *et al.*, 1994). Walther et al., (1996) isolated Sertoli cells from testes of postnatal day 10 H-2K^b-tsA58 transgenic mice and established a number of immortalised Sertoli cell lines containing the H-2K^b-tsA58 TAg gene. One of the Sertoli cell lines, SK11, was shown to display the pattern of cytoskeletal markers characteristic for Sertoli cells in vivo. Although not all Sertoli cell morphological characteristics had been conserved the SK11 cell line does express mRNA for inhibin-α, SGP-2, transferrin, SF-1, aromatase, GATA-1 and AR (Walther *et al.*, 1996; Walther *et al.*, 1997).

In the present chapter gene expression in the SK11 Sertoli cell line was further investigated focusing in particular on effects of T and E on GATA-4, Musashi-1 and inhibin-α gene expression (re: previous chapters). It was hoped that in using an immortalised Sertoli cell line previous problems encountered using primary Sertoli cell cultures would be removed allowing direct effects of E and T on Sertoli cell behaviour to be assessed.

6.2. Materials and Methods

6.2.1. Immortal Mouse Sertoli cell line (SK11)

An immortal Sertoli cell-line (SK11) established from H-2K^b tsA58 transgenic mice carrying the inducible temperature-sensitive SV-40 T antigen (Walther *et al.*, 1996) was a gift from Dr Walther, Hamburg. SK11 cells were maintained in Dulbecco's modified Eagle's media (DMEM) with 4500mg

glucose, glutamine, pyridoxine.HCl and sodium bicarbonate (Sigma) containing 10% fetal bovine serum (Gibco-BRL), 50 Units/ml penicillin/streptomycin (Gibco-BRL) and 2.5μg/ml amphotericin B (Sigma) at 33°C, 5% CO_2 and 95% humidity. When cell confluency had reached 70% at 33°C, some flasks were placed in a 39°C incubator at 5% CO_2 and 95% humidity for 48 hours whilst cells growing in flasks that had remained at 33°C reached 100% confluency (approximately $1x10^6$ cells/75cm^2 flask).

6.2.1.1. Treatments

Treatments, stock solutions and working concentrations were as given in section 5.2.1.2. 100μg of testosterone (Sigma, 4-androsten-17β-ol-3-one) (T) was dissolved in 1ml absolute ethanol producing a stock solution at 100μg/ml and appropriate volumes were added to cell culture media to give a final working solution of 100ng/ml. A stock solution of oestradiol benzoate (Sigma) (E) in absolute ethanol (10μg/ml) was added to cell culture media such that a final working concentration of 10ng/ml was produced. Equivalent volumes of absolute ethanol were added to control media. Forskolin (Sigma) was dissolved in DMSO to give a stock solution at 10mM. Appropriate volumes were added to media to give a working concentration of 10μM.

Treatments were timed from the point at which SK11 cells were placed into the 39°C incubator such that 48 hour treatments were added at this point, 24 hour treatments were administered 24 hours later and 4 and 1 hour treatments were carried out 44 and 47 hours after placement at the non-permissive temperature, respectively. Therefore all SK11 cells were grown in culture for identical amounts of time. Control cells grown at 33°C were administered equivalent treatment regimes. Cells were harvested 48 hours after placement in the 33°C or 39°C incubators. When steroid treatments were added DMEM media containing 10% charcoal stripped fetal calf serum was used in place of normal fetal calf serum.

6.2.2. RNA extraction and separation

Total RNA was extracted from whole mouse and rat tissues as described in Chapter 2.

6.2.2.1. Lysis of cultured SK11 cells

After 48 hours in culture at 33°C or 39°C media were aspirated and cells were washed with PBS. 1ml of 1x trypsin in DMEM (Sigma) was added to the cells to detach them from the culture flask surface with gentle agitation. Once the majority of cells were detached, 5mls of DMEM was added and the cell suspension was placed in a 15ml centrifuge tube (Corning) and cells were gently pelleted at 4°C for 10 minutes at 3000g. The supernatant was carefully removed and the cell pellet was snap frozen prior to storing at –70°C.

6.2.2.2. RNA extraction from frozen SK11 cells

Whilst frozen SK11 cell pellets were thawing on ice, 1ml of Tri-Reagent™ was added to lyse the cells and extract RNA. RNA extraction was carried out as described in Chapter 2 with appropriate adjustment of volumes.

6.2.2.3. RNA separation

10μg of total RNA extracted from cultured SK11 cells and whole rat and mouse tissues was separated on 1.5% denaturing agarose gels and blotted onto nitrocellulose membranes as described in Sections 2.3.3. and Section 2.9, respectively. RNA was extracted from SK11 cells in each treatment group. Experiments were duplicated and RNA from each experiment was separated alongside total RNA extracted from day 18 whole rat testes, rat kidney and day 10 mouse testes which were used as control RNA samples.

6.2.3. Northern blot analysis

Northern blot analysis of total RNA was carried out as described in Section 2.9 and cDNA probes for SGP-1, inhibin-α and 18S were as described in Chapter 3. Musashi-1 cDNA probe was amplified as described in Maguire et al (1999) and section 3.2.1.3. except that the partial Musashi-1 cDNA was amplified by PCR from a pool of day 10 mouse testis cDNAs prepared by RT using oligo dT primers as described in section 2.5.2. Partial cDNAs for GATA-4 and GATA-1 were amplified by PCR as described in section 3.2.1.4

and 3.2.1.5, respectively. Both partial cDNAs were amplified from a pool of SK11 cell (grown at 39°C) cDNAs prepared by RT using oligo dT primers as described in section 2.5.2. The SK11 GATA-4 partial cDNA had 100% sequence homology with the published mouse GATA-4 cDNA over the region of sequence whilst the SK11 GATA-1 partial cDNA sequence had 95.8% homology with the published rat GATA-1 sequence over the region of sequence. Radiolabelling of cDNA probes was carried out following the protocol given in Chapter 2. Quantification of radiolabelled signals was carried out as described in Section 2.9.

6.2.4. Immunocytochemistry

6.2.4.1. Mouse testes tissue sections

Whole mouse testes were fixed, embedded in paraffin and sectioned as described in section 2.4. Immunocytochemistry using antibodies raised against PCNA, SGP-1, inhibin-α, Musashi-1, GATA-1, GATA-4, AR and ERβ was performed on fixed mouse tissue sections as described in Chapter 3.

6.2.4.2. SK11 cultured cells

SK11 cells were grown on glass chamber slides containing one or four culture wells (Gibco). Treatment of SK11 cells was carried out as described in section 6.2.1.1. and different timed treatments were set up on the same slide as shown below (Figure 6.1.)

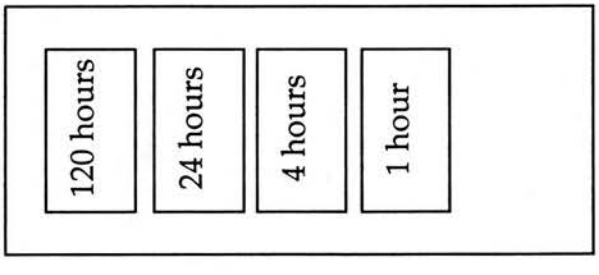

Figure 7.1: Diagram to show set up of timed treatment of SK11 cells cultured on 4 chamber glass slides. SK11 cells were cultured in each chamber. At appropriate time points the media in each chamber were removed and replaced with media containing ethanol, F (10μM), F+E (10ng/ml) or F+T (100ng/ml).

SK11 cells grown on glass slides were fixed and prepared for immunocytochemical analysis as described in section 5.1.4.

Haemotoxylin and eosin (H and E)

H and E staining of control SK11 cells required a 1-3 minute incubation in haemotoxylin followed by a wash in distilled water and a short incubation in Scott's tap water. If the haemotoxylin staining was too intense, it was reduced by placing the slides in 1% acid alcohol for 5-10 seconds and once the blue haemotoxylin stain was optimal the slides were placed for 5-20 seconds in 1% eosin, washed in tap water and quickly dehydrated in an increasing series of alcohols as described in Chapter 2.

Sertoli cell proteins

Immunolocalisation of GATA-4 to fixed SK11 cells was performed as described in Chapter 5. ERβ was immunodetected in SK11 cells as stated in Chapter 5 except that in place of permeabilising the cells with NP40 solution a 5 minute incubation in 0.1% sodium dodecyl sulfate (SDS) in PBS was carried out followed by 3 x 5 minute PBS washes as reported in Brown et al., (1996) (Brown *et al.*, 1996). Blocking serum was then added and the immunocytochemical procedure was continued as in Chapter 5.

6.3. Results

6.3.1. Protein expression in postnatal mouse testis

SGP-1 (Fig. 6.2.a) and inhibin-α (Fig. 6.2.b) were immunolocalised to Sertoli cell cytoplasm in postnatal day 10 mouse testes. Both Sertoli cell nuclei and cytoplasm were immunostained for Musashi-1 protein in day 10 mouse testes (Fig. 6.2.c). Nuclear immunostaining for Musashi-1 was of a greater intensity than cytoplasmic Musashi-1 immunostaining, GATA-4 was expressed in Sertoli and interstitial cell nuclei (Fig. 6.2.d). Stage specific immunoexpression of all four Sertoli cell proteins was not observed in mouse testes at this age.

GATA-1 was not immunoexpressed in postnatal day 3 mouse testes (Fig.6.3.a). In day 8 mouse testes GATA-1 protein was immunolocalised to Sertoli cell nuclei in some seminiferous tubules where Sertoli cell nuclei were closer to the basement membrane of the seminiferous epithelium (Fig.6.3.c). The intensity of GATA-1 immunostaining was increased in Sertoli cell nuclei by day 10 and day 12 (Fig. 6.3.e, g). The majority of cells, including Sertoli cells (black arrowheads), in day 3 mouse testes were immunopositive for PCNA (Fig.6.3.b). By day 8 a large number of spermatogonia were still immunopositive for PCNA as were some Sertoli cell nuclei (black arrowheads). However a number of Sertoli cell nuclei were immunonegative for PCNA (black arrows) (Fig. 6.3.d). In day 10 mouse testes PCNA was immunoexpressed in primary spermatocytes whilst the majority of Sertoli cell nuclei were immunonegative for PCNA (black arrows). A small number of immunopositive Sertoli cell nuclei were still present in mouse testes at this age (black arrowhead) (Fig. 6.3.f). However by day 12 all Sertoli cell nuclei were immunonegative for PCNA (black arrows) and the number of spermatogonia and spermatocytes positively immunostained for PCNA had increased (Fig. 6.3.h).

AR immunoexpression was restricted to interstitial cell nuclei in day 2 mouse testes (Fig. 6.4.a). By day 8, AR was also immunolocalised to Sertoli cell nuclei (Fig.6.4.b). The intensity of Sertoli cell and interstitial cell nuclear immunostaining was increased in day 10 mouse testis (Fig.6.4.c). AR was immunolocalised to Sertoli and interstitial cell nuclei from day 12 (Fig. 6.4.d-f) and into adulthood where AR immunoexpression in Sertoli cells was stage specific (Fig.6.4.g).

In day 2 and day 3 mouse testes ERβ was immunolocalised to interstitial cells and cells within the seminiferous tubules (Fig.6.5.a, b). The intensity of ERβ immunostaining in Sertoli cell nuclei was increased in day 10 mouse testes and both interstitial and Sertoli cell nuclei remained positively immunostained throughout development and into adulthood (Fig. 6.5.c-g). Between days 10 and 12 developing spermatogonia were found to express ERβ (Fig. 6.5. c, d) and by day 16 primary spermatocytes are also

immunopositive for ERβ (Fig. 6.5. f). In adult mouse testes (Fig. 6.5g) round spermatids also express ERβ. Some non-specific immunostaining was observed in the adult mouse testes when preabsorbed antibody was used in place of primary antibody (Fig. 6.5.h).

6.3.2. Steroid regulation of mRNA and protein expression in SK11 cells

The nuclei of SK11 cells cultured at 33°C appeared to contain more condensed chromatin (blue arrows) than those grown at 39°C (black arrows). In addition more mitotic figures (black arrowheads) were observed at the permissive (Fig. 6.6.b) temperature than within SK11 culture at 39°C (Fig. 6.6.d).

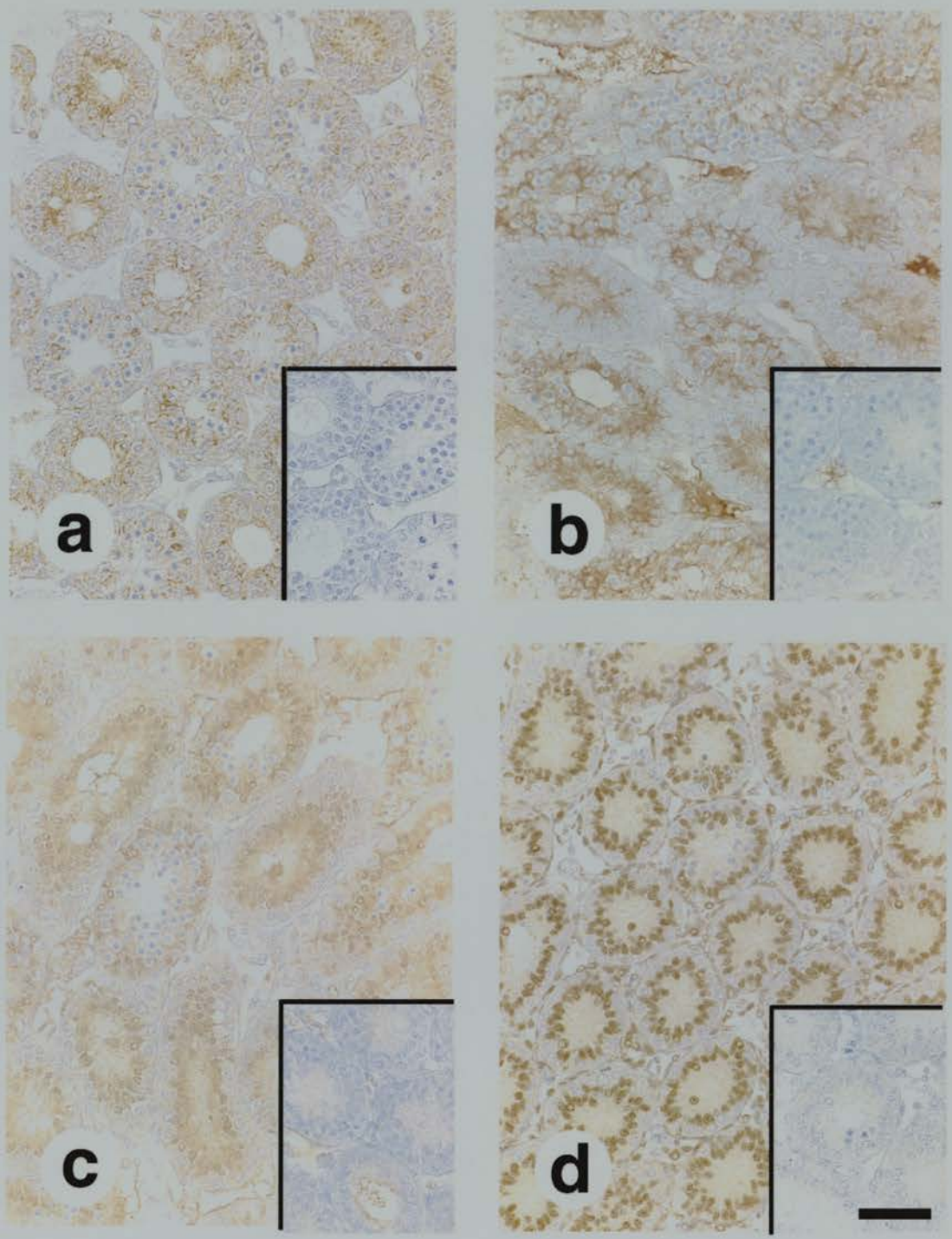

Figure 6.2: Immunolocalisation of SGP-1, inhibin-α, Musashi-1 and GATA-4 to postnatal day 10 mouse testes. Sertoli cell proteins SGP-1 **(a)**, inhibin-α **(b)**, Musashi-1 **(c)** and GATA-4 **(d)** were immunolocalised to testes of postnatal day 10 mice. Corresponding negative controls where primary antibody was replaced with normal rabbit IgGs **(a)**, normal mouse IgGs **(b)**, normal rat IgGs **(c)** and normal goat IgGs **(d)** are shown in insets. Scale bar represents 50μm.

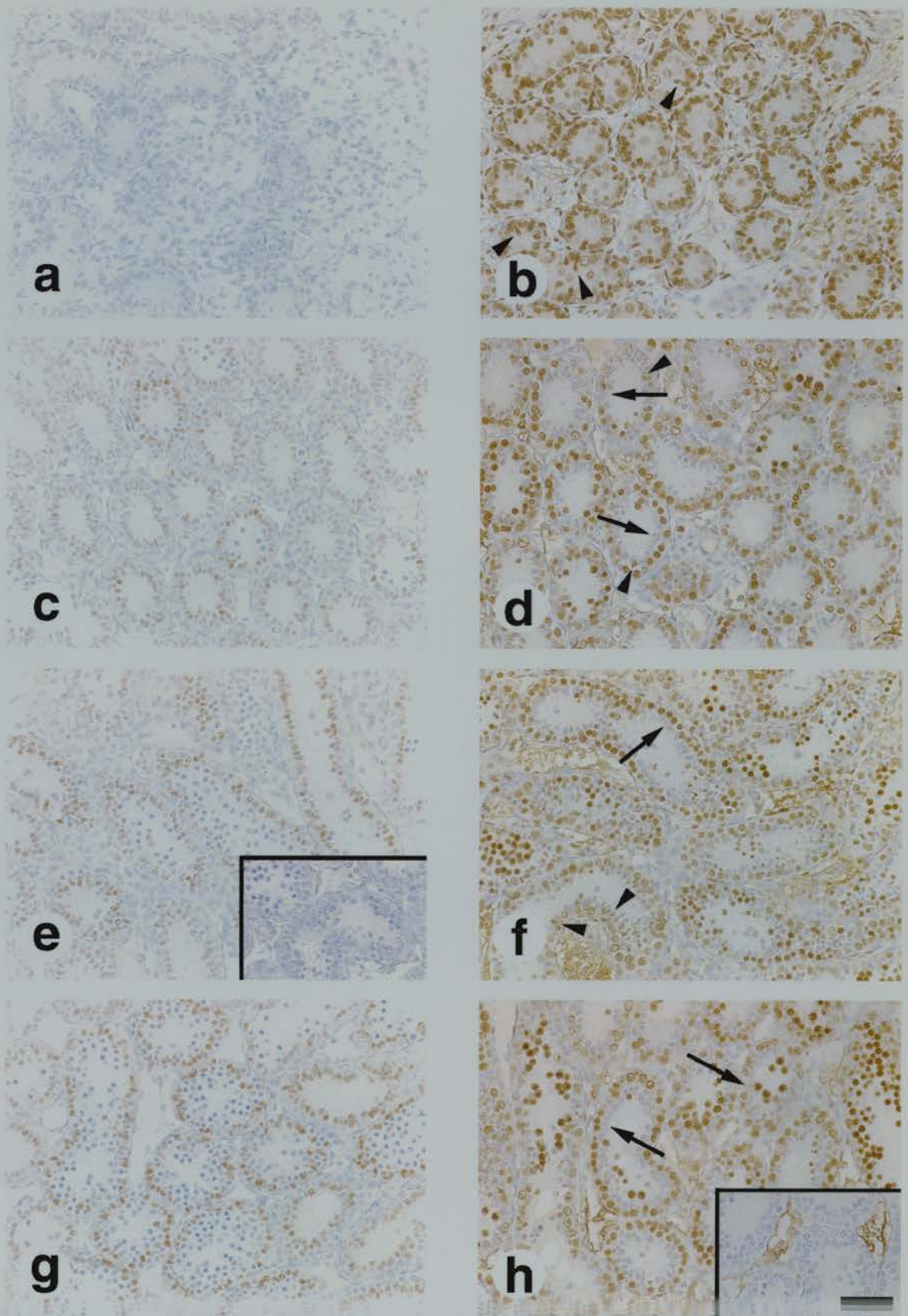

Figure 6.3: Immunolocalisation of GATA-1 and PCNA to postnatal mouse testis. GATA-1 **(a,c,e,g)** and PCNA **(b,d,f,h)** were immunolocalised to testes from mice aged postnatal day3**(a,b)**, day 8**(c,d)**, day 10**(e,f)** and day 12**(g,h)**. Arrows point to immunonegative Sertoli cell nulcei and arrowheads point to immunopositive Sertoli cell nulcei. Insets show negative controls where primary antibody was replaced with normal rat IgGs **(e)** and normal mouse IgGs **(h)**.Scale bar represents 50µm

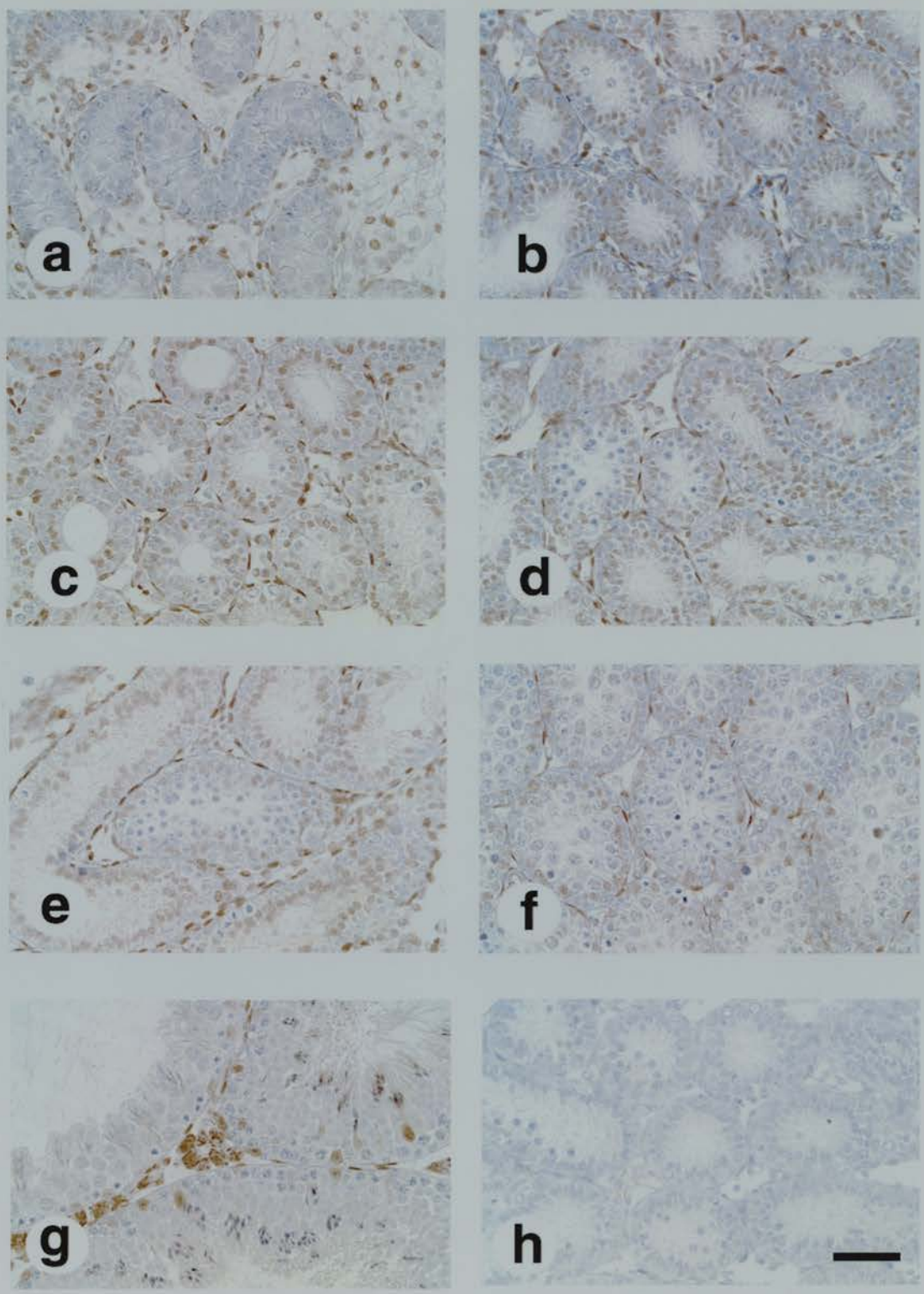

Figure 6.4: Immunolocalisation of androgen receptor (AR) to postnatal mouse testes. AR was immunolocalised to testes from mice aged postnatal day 2 **(a)**, day 8 **(b)**, day 10 **(c)**, day 12 **(d)**, day 14 **(e)**, day 16 **(f)** and adult **(g)**. A negative control from day 10 testes is shown in **(h)** when primary antibody was replaced with normal rabbit IgGs. Scale bar represents 50μm.

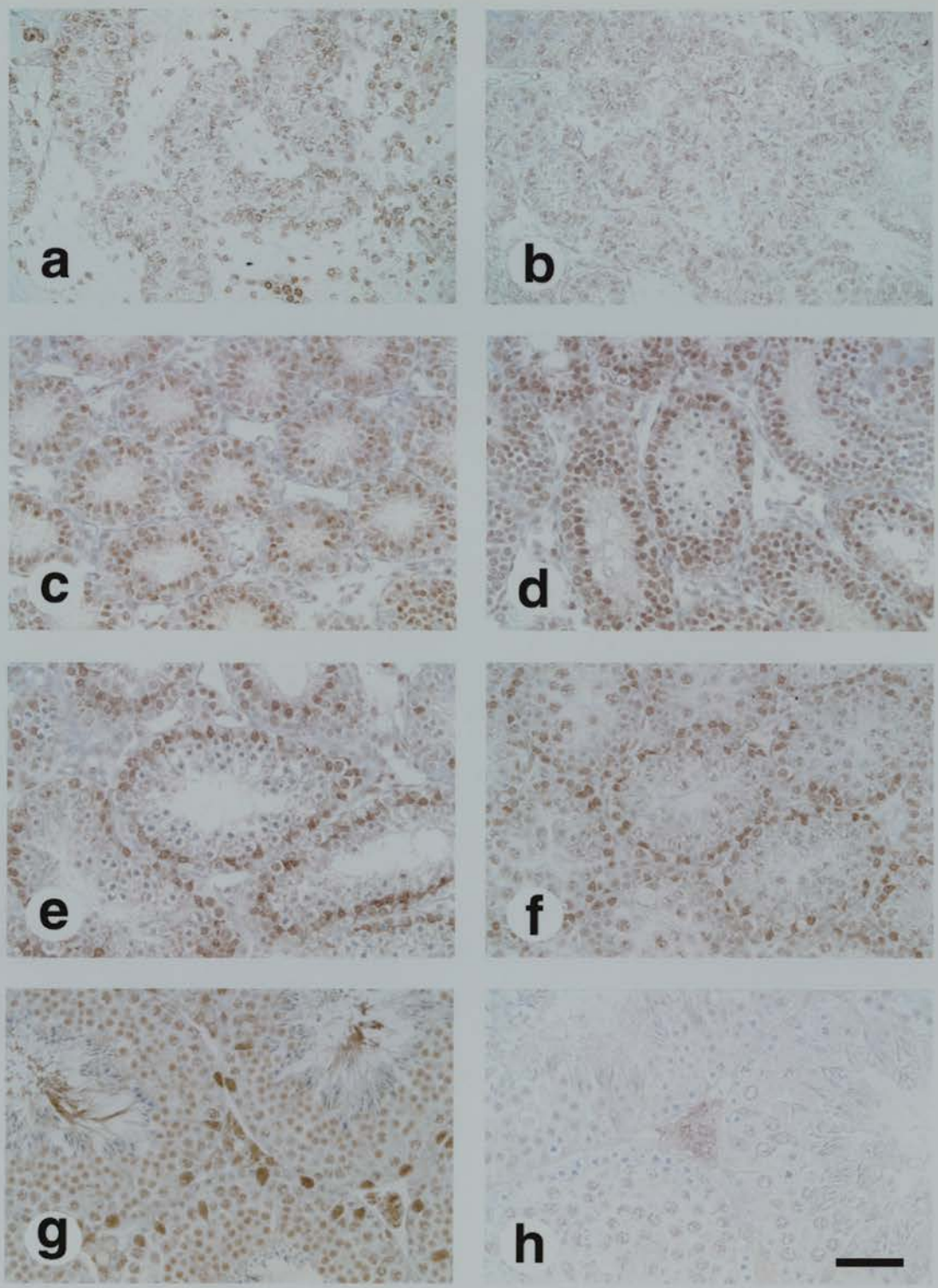

Figure 6.5: Immunolocalisation of oestrogen receptor-β (ERβ) to postnatal mouse testes. ERβ was immunolocalised to testes from mice aged postnatal day 2 **(a)**, day 3 **(b)**, day 10 **(c)**, day 12 **(d)**, day 14 **(e)**, day 16 **(f)** and adult **(g)**. A negative control on adult testes is shown in **(h)** when primary antibody was replaced with preabsorbed antibody. Scale bar represents 50μm.

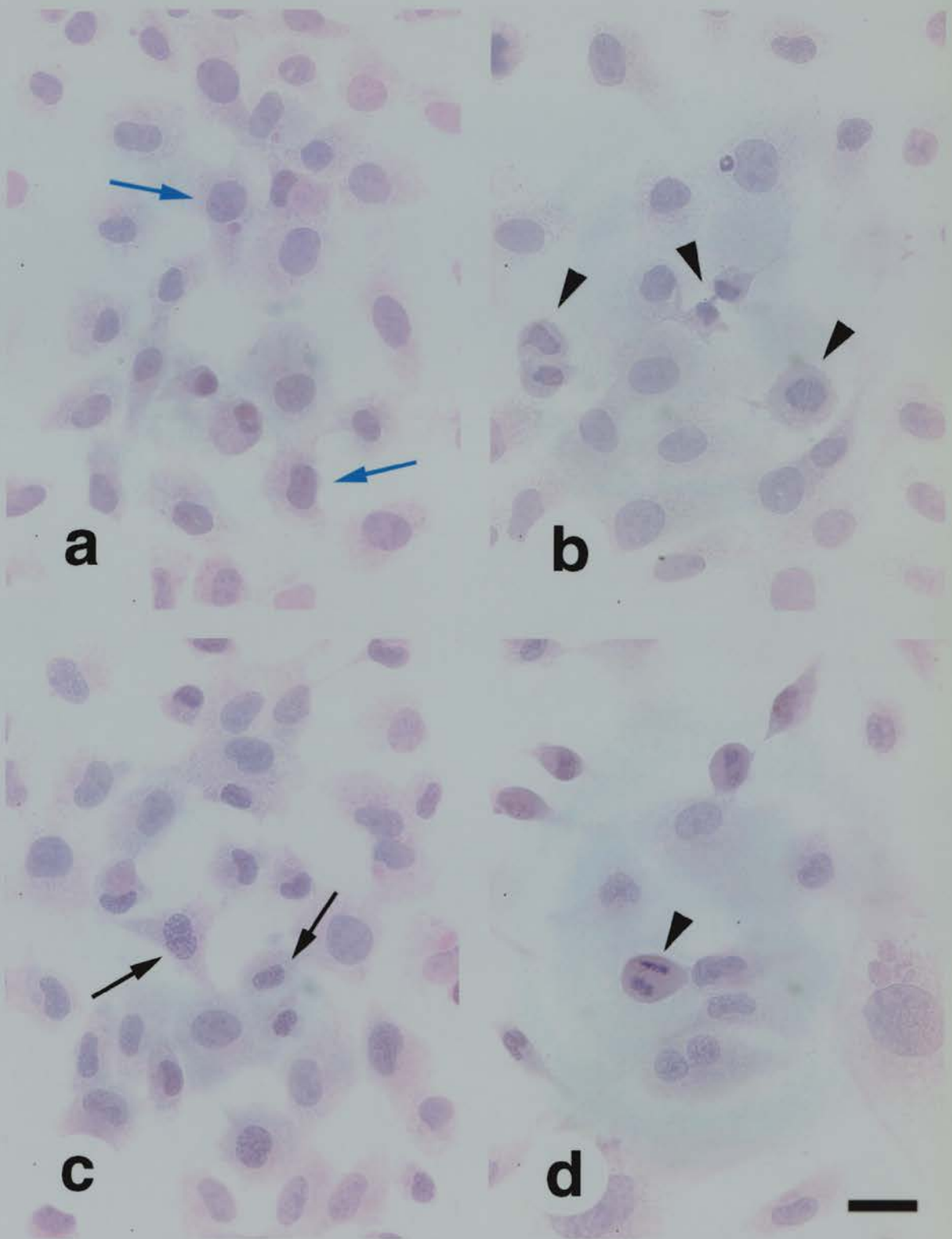

Figure 6.6: Haemotoxylin and eosin (H+E) staining of control SK11 cells cultured at both 33°C and 39°C. SK11 cells grown on glass slides at 33°C **(a, b)** and 39°C **(c, d)** in control media were stained with H and E. Blue arrows point to nuclei containing condensed chromatin, black arrows point to nuclei containing less condensed chromatin and black arrowheads point to mitotic figures. Scale bar represents 20μm.

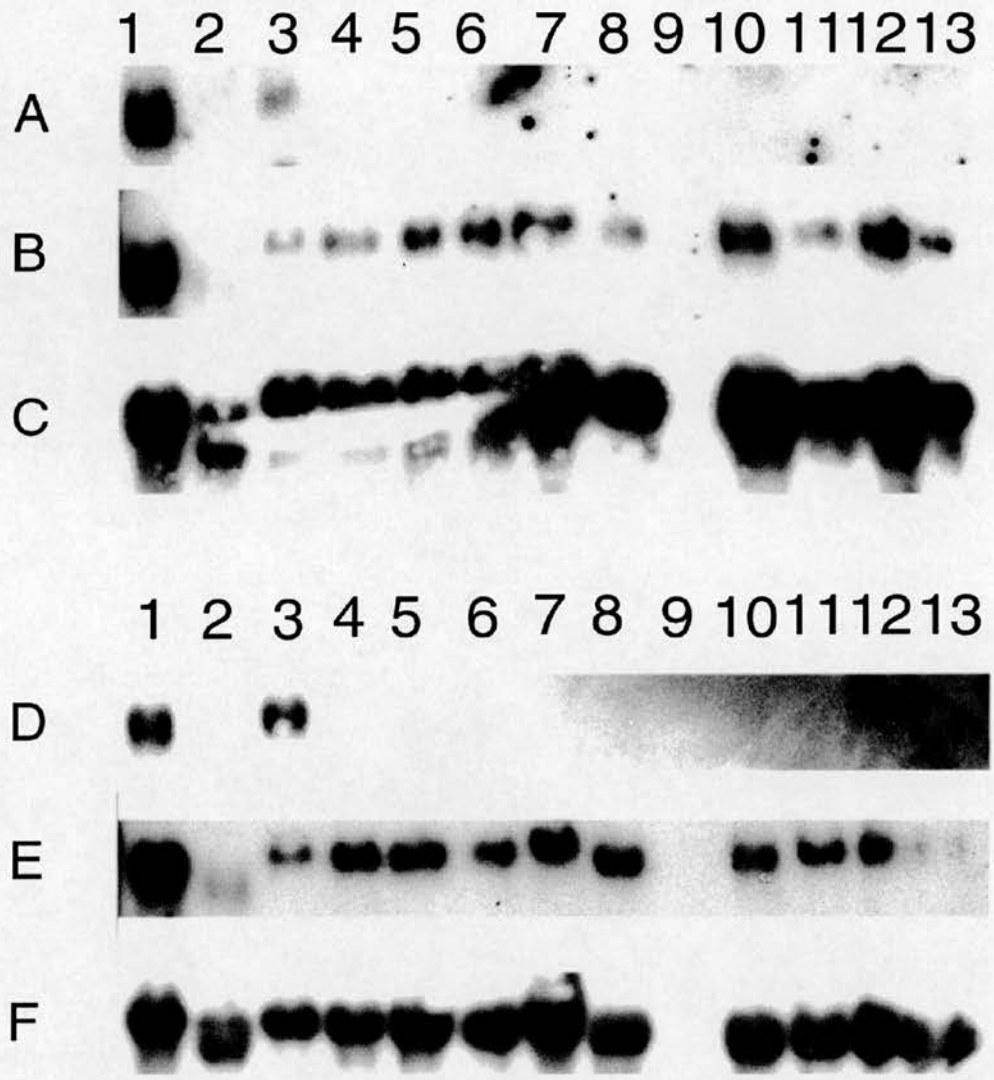

Figure 6.7: Northern blot analysis of inhibin-α and Musashi-1 mRNA expression in SK11 cells. Northern blot analysis detected a single 1.5kb inhibin-α mRNA transcript**(A)**, a single 3kb Musashi-1 mRNA transcript **(D)**, a 2.6kb SGP-1 mRNA transcript **(B, E)** and a 1.9kb 18S rRNA transcript **(C, F)**. 10μg of total RNA was loaded per lane and RNA was obtained from postnatal day 18 rat testes **(1)**, rat kidney **(2)**, postnatal day 10 mouse testes **(3)**, SK11 39°C; F **(4)**, SK11 39°C; C **(5)**, SK11 33°C; F **(6)**, SK11 33°C; C **(7)**, SK11 39°C; T+F 24 hours **(8)**, SK11 39°C; T+F 4 hours **(9)**, SK11 39°C; T+F 1 hour **(10)**, SK11 39°C; E+F 24 hours **(11)**, SK11 39°C; E+F 4 hours **(12)** and SK11 39°C; E+F 1hour **(13)**. C = ethanol, F = forskolin (10μM), E = oestradiol benzoate (10ng/ml) and T = testosterone (100ng/ml).

A single 1.5kb inhibin-α mRNA transcript was detected in day 18 rat testes and day 10 mouse testes RNA samples (Fig. 6.7). The levels of inhibin-α mRNA in all SK11 cell RNA samples were too low to be detected by Northern blot analysis (Fig. 6.7. A). Musashi-1 cDNA hybridised to a 3 kb mRNA transcript in day 18 rat and day 10 mouse testes RNA samples. However levels of Musashi-1 mRNA in all SK11 cell RNA samples were too low to be detected by Northern blot analysis (Fig. 6.7. D). All RNA samples were reprobed with radiolabelled SGP-1 and 18S cDNA probes which detected a 2.6kb SGP-1 mRNA transcript (Fig. 6.7.B, E) and a 1.9kb 18S rRNA transcript (Fig. 6.7.C, F) in all RNA samples analysed.

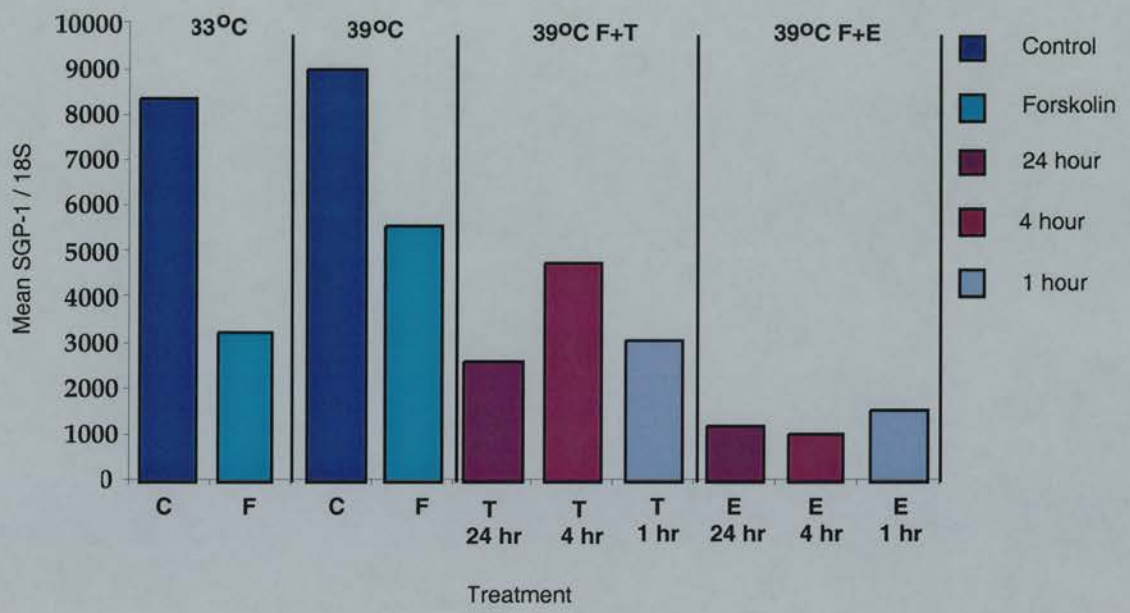

Figure 6.8: Changes in SGP-1 mRNA expression following steroid treatment of SK11 cells. Hybridisation signals for SK11 SGP-1 mRNA levels were normalised against corresponding 18S rRNA. The results shown are the mean SGP-1:18S values from two separate experiments.

To determine if steroid treatment had any regulatory effect on SK11 SGP-1 expression, hybridisation signals for SGP-1 mRNA from 2 separate experiments were normalised against corresponding 18S values. Mean SGP-1:18S values were calculated and results are shown in Fig. 6.8. F treatment at both 33°C and 39°C downregulated SGP-1 mRNA expression. At 39°C, administration of E and T in the presence of F further downregulated SGP-1 gene expression and the decline in gene expression was greatest following E

administration. The extent of downregulation in expression of SGP-1 in the E treated SK11 cells was unchanged across timed treatments. In contrast 24 and 1 hour T administration had a greater effect on SGP-1 expression than the 4 hour T treatment.

Using a radiolabelled GATA-4 cDNA probe, a single 3.1kb mRNA transcript was detected in all SK11 and whole testes RNA samples analysed (Fig. 6.9.A). A 2.6kb SGP-1 mRNA transcript (Fig. 6.9.B) and a 1.9kb 18S rRNA transcript (Fig. 6.9.C) were also detected in all RNA samples.

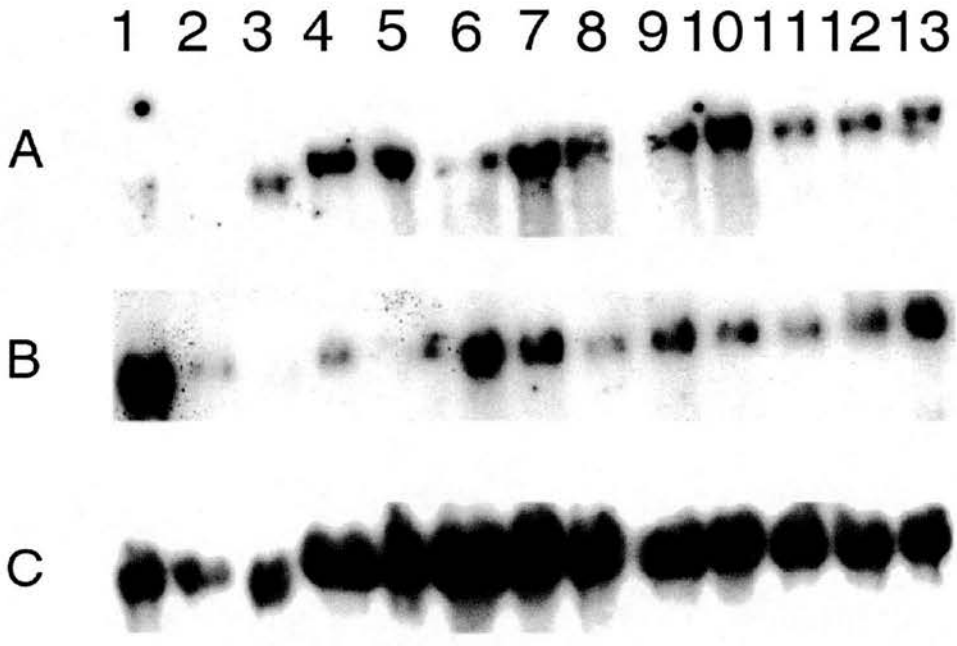

Figure 6.9: Northern blot analysis of GATA-4 mRNA expression in SK11 cells. A single 3.1kb GATA-4 mRNA transcript **(A)** was hybridised to 10µg of total RNA obtained from postnatal day 18 rat testes **(1)**, rat kidney **(2)**, postnatal day 10 mouse testes **(3)**, SK11 cells cultured at; 39°C +F **(4)**; 39°C +C **(5)**, 33°C +F **(6)**; 33°C +C **(7)**; 39°C F+T 24 hours **(8)**; 39°C F+T 4 hours **(9)**; 39°C F+T 1 hour **(10)**; 39°C F+E 24 hours **(11)**; 39°C F+E 4 hours **(12)** and 39°C F+E 1 hour **(13)**. The RNA blot was stripped and reprobed with SGP-1 and 18S cDNA probes which detected a 2.6kb SGP-1 mRNA transcript **(B)** and a 1.9kb 18S rRNA transcript **(C)**. C = ethanol, F = forskolin (10µM), T = testosterone (100ng/ml) and E = oestradiol benzoate (10ng/ml).

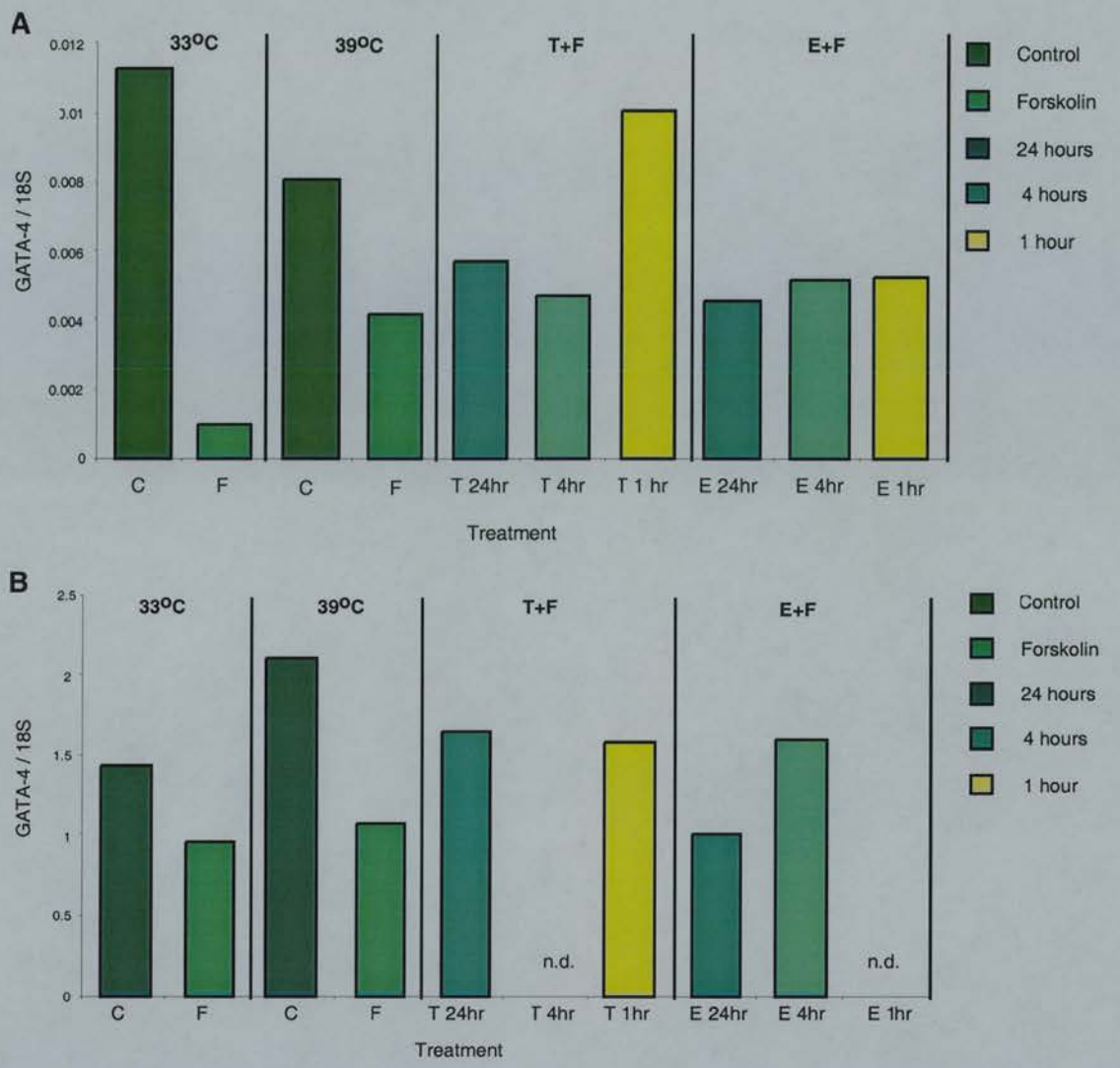

Figure 6.10: Changes in GATA-4 mRNA expression following steroid treatment of SK11 cells. GATA-4 mRNA hybridisation signals from two separate experiments were quantified and normalised against corresponding 18S rRNA values. In **(B)**, the T 4 hour RNA sample was missing and an 18S value for E 1 hour sample could not be obtained.

Hybridisation signals from GATA-4 and 18S Northern blot analyses were quantified and GATA-4 mRNA expression by SK11 cells was normalised against corresponding 18S rRNA values. Results from two separate duplicate experiments are shown in Fig. 6.10. In both experiments F treatment decreased GATA-4 mRNA expression in SK11 cells cultured at both 33°C and 39°C. There were some inconsistencies regarding the effect of E and T treatment on SK11 cell GATA-4 gene expression. Overall it appeared that both steroids had no real effect on GATA-4 gene expression at

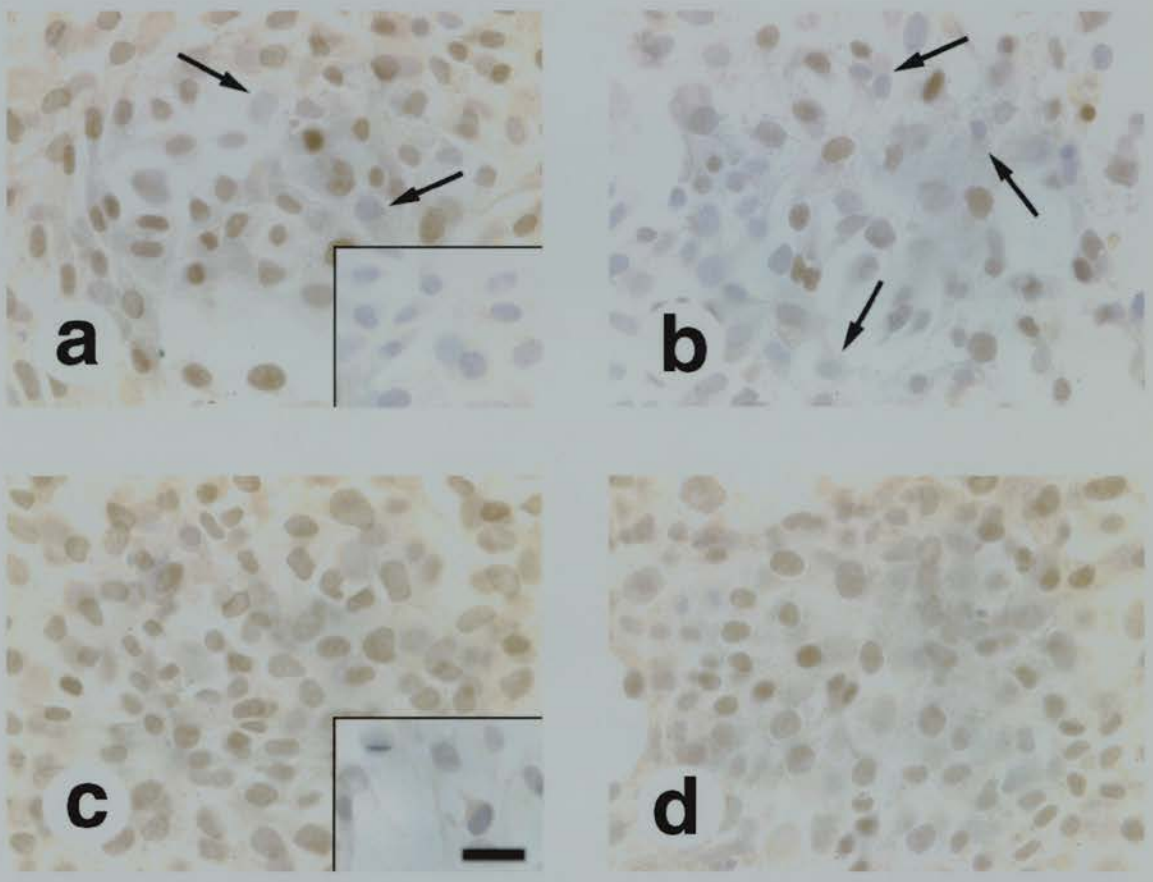

Figure 6.11: Immunolocalisation of GATA-4 and oestrogen receptor-β (ERβ) in SK11 cells grown at 33°C. GATA-4 was immunolocalised to SK11 cells cultured at 33°C in the presence **(b)** and absence **(a)** of forskolin (10μM). ERβ was immunolocalised to SK11 cells grown at 33°C in the presence **(d)** and absence **(c)** of forskolin (10μM). Negative controls when primary antibody was replaced with normal goat IgGs **(a)** and preabsorbed ERβ antibody **(c)** are shown in insets. Arrows point to immunonegative SK11 cell nuclei following GATA-4 immunolocalisation. Scale bar represents 20μm.

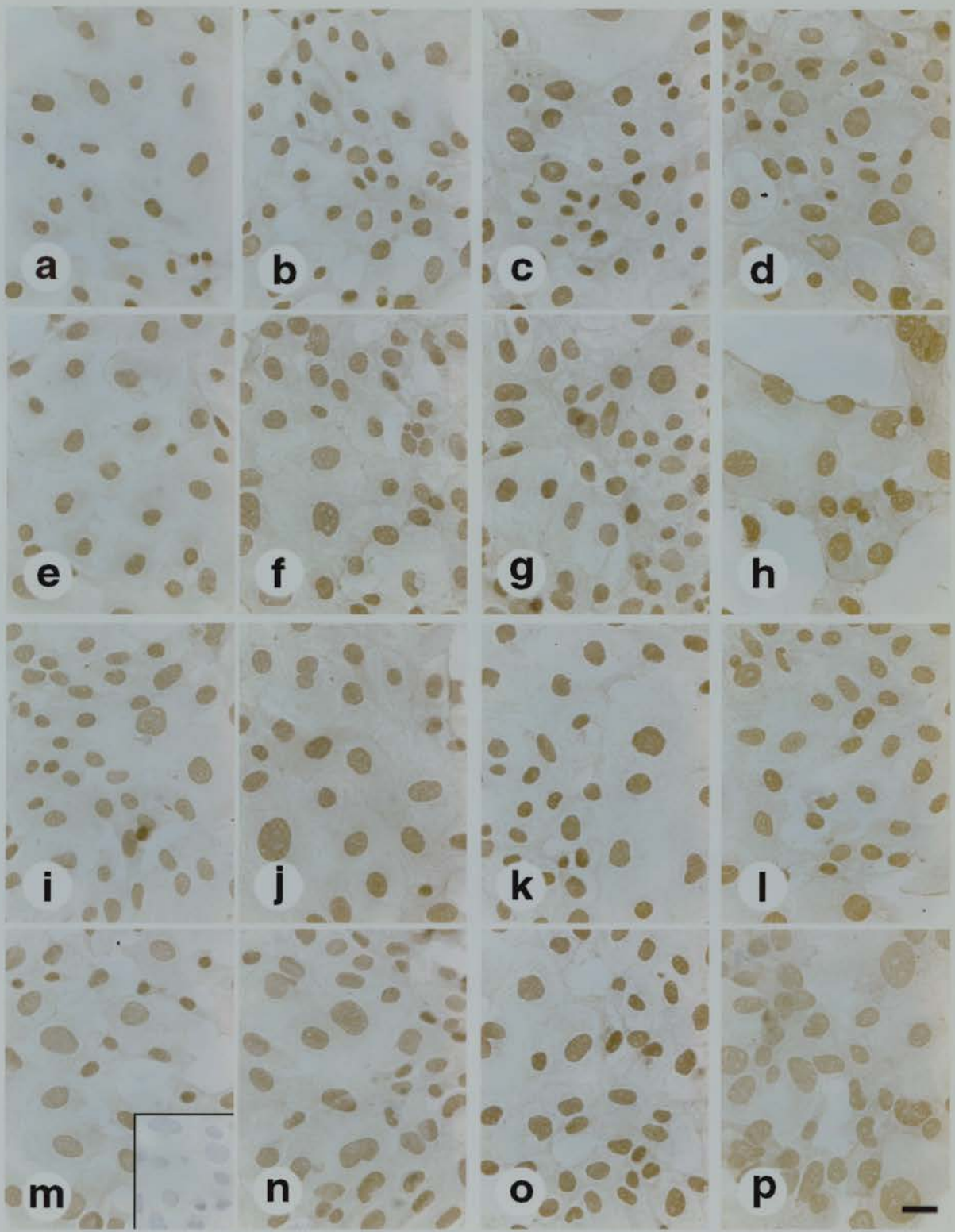

Figure 6.12: Immunolocalisation of oestrogen receptor (ERβ) to SK11 cells. ERβ was immunolocal--ised to SK11 cells grown at 39°C in the presence of ethanol **(a, e, ,i, m)**, forskolin (F) at 10μM **(b, f, j, n)**, F and oestrogen benzoate (E) at 10ng/ml **(c, g, k, o)** and F and testosterone (T) at 100ng/ml **(d, h, l, p)**. SK11 cells were treated for 48 hours **(a-d)**, 24 hours **(e-h)**, 4 hours **(i-l)** and 1 hour **(m-p)**. Negative control when primary antibody was replaced with preabsorbed ERβ antibody is shown in inset. Scale bar represents 20μm.

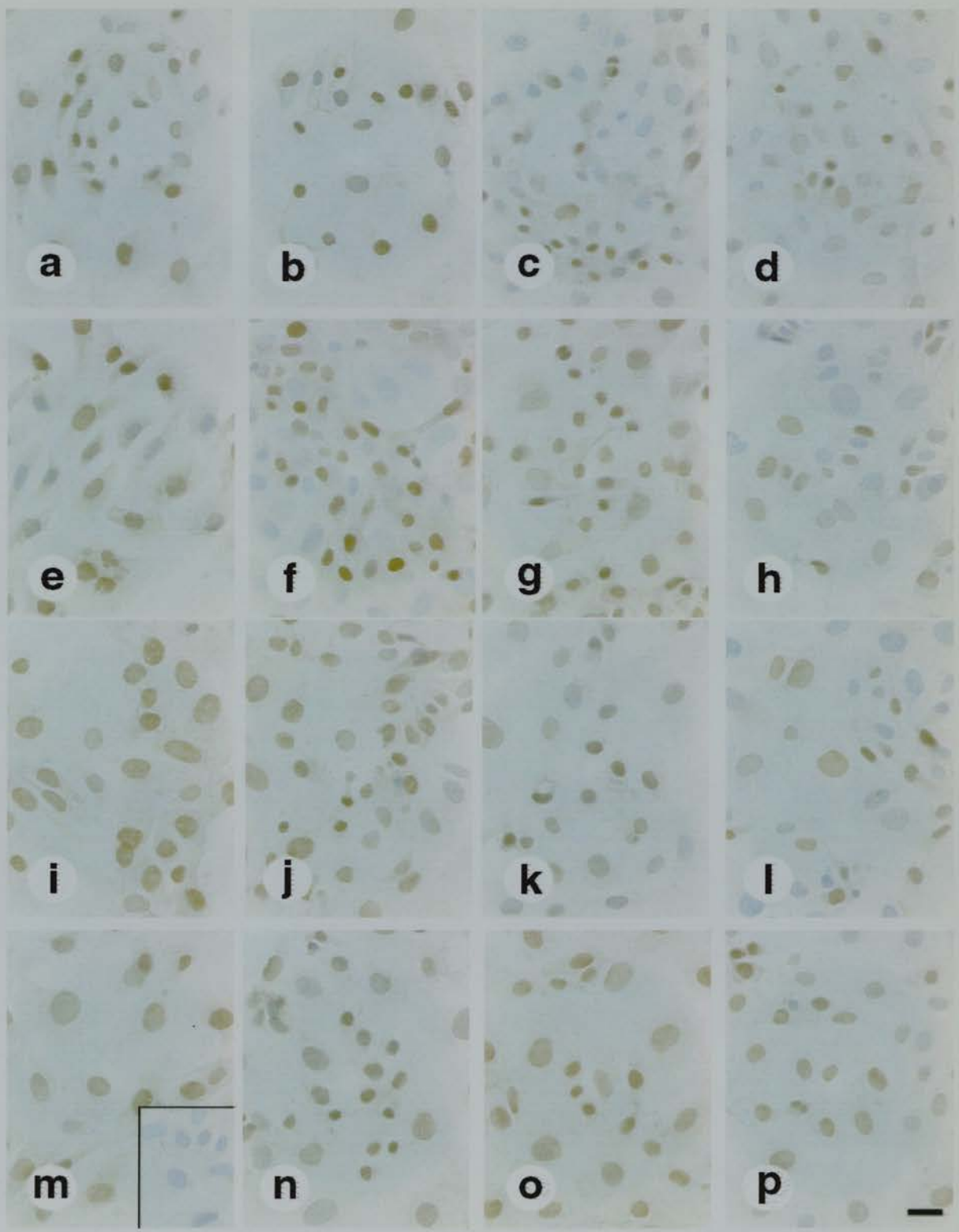

Figure 6.13: Immunolocalisation of GATA-4 to SK11 cells. GATA-4 was immunolocalised to SK11 cells grown at 39°C in the presence of ethanol **(a, e, i, m)**, forskolin (F) at 10μM **(b, f, j, n)**, F and oestradiol benzoate (E) at 10ng/ml **(c, g, k, o)**, F and testosterone (T) at 100ng/ml **(d, h, l, p)**. SK11 cells were treated for 48 hours **(a-d)**, 24 hours **(e-h)**, 4 hours **(i-l)** and 1 hour **(m-p)**. Negative control when primary antibody was replaced with normal goat IgGs is shown in inset. Scale bar represents 20μm.

all time points assessed apart from an increase in gene expression following 1 hour F+T treatment and 4 hour F+E treatment in one of the two experiments.

GATA-4 and ERβ proteins were immunolocalised to nuclei of SK11 cells grown at 33°C (Fig. 6.11.). Following 24 hour F treatment the number of SK11 cell nuclei positively immunostained for GATA-4 was decreased when compared to control SK11 cells (Fig. 6.11.a, b; immunonegative SK11 nuclei indicated by blackarrows). In contrast the number of ERβ positively immunostained SK11 nuclei was unchanged following F administration (Fig. 6.11.c, d).

At 39°C all SK11 cell nuclei were immunopositive for ERβ protein (Fig. 6.12.). There was no change in the level of ERβ immunoexpression in SK11 cells following F treatment when compared to control SK11 cells (Fig. 6.12. b, f, j, n). Intensity of ERβ immunostaining was greater in SK11 cells following all F+E timed treatments (Fig. 6.12.c, g, k, o). Following F+T treatment for 48, 24 and 4 hours there was an increase in the intensity of immunostaining of ERβ in SK11 cells (Fig. 6.12.d, h, l).

GATA-4 was specifically immunolocalised to the nuclei of SK11 cells in culture at 39°C (Fig. 6.13.). Some SK11 cells were immunonegative for GATA-4. No difference in intensity of GATA-4 immunostaining could be observed between control (Fig. 6.13. a, e, i, m), F+E (Fig. 6.13. c, g, k, o) and F+T (Fig. 6.13. d, h, l, p) treated SK11 cells. SK11 cells treated with F alone for 48 and 24 hours had a greater intensity of GATA-4 immunostaining when compared to corresponding control SK11 cells (Fig. 6.13. b, f).

6.4. Discussion

In the study presented the SK11 cell line appeared morphologically similar to SK11 cells described in Walther et al., (1996). Nucleoli were more apparent in nuclei of cells grown at 33°C than at 39°C and more mitotic figures could be observed at 33°C in accordance with proliferation of the cell line at the

permissive temperature. Mitotic figures were also observed at 39°C. Thus a very small number of SK11 cells are actually still dividing after 48 hours at the non-permissive temperature and this was also reported by Walther et al., (1996) (Walther *et al.*, 1996). However Walther et al., (1996) could not detect proliferating SK11 cells after 96 hours of culture at the non-permissive temperature. Following H and E staining, nuclei of SK11 cells at 39°C appeared to contain less-condensed chromatin when compared to cells grown at 33°C suggesting that SK11 cells undergoing differentiation at 39°C are more transcriptionally active than proliferating cells at 33°C.

The SK11 cell line originated from Sertoli cells isolated from day 10 H-2K^{b}-tsA58 transgenic mice. Analysis of GATA-1 and PCNA protein expression during mouse testis development (shown in the present study) suggested that mouse Sertoli cells cease proliferation between days 8 and 12 of development. Hence by day 10, the majority of Sertoli cells have ceased dividing and are undergoing maturation and consequently mouse Sertoli cells at this age are the equivalent of day 18 Sertoli cells in the rat testis (Chapter 4). Under normal conditions, day 10 mouse Sertoli cells express SGP-1, inhibin-α, Musashi-1, GATA-1 and GATA-4 proteins. In addition both AR and ERβ proteins are expressed in Sertoli cell nuclei at this stage of mouse testis development. Expression levels of inhibin-α and Musashi-1 in SK11 cells were too low to be detected by Northern blot analysis and immunocytochemistry, although previously the more sensitive technique of RTPCR analysis detected inhibin-α mRNA expression in SK11 cells (Walther *et al.*, 1996). No previous research has been carried out to investigate Musashi-1 expression in the SK11 cell line and therefore it is not known if Musashi-1 expression has been lost in the immortalised cell line as has been shown to occur with other proteins during cell culture (Angus *et al.*, 1993; Sutton *et al.*, 1998) or if mRNA expression levels are just too low to be detected using Northern blot analysis. RPAs and RTPCR will need to be carried out to determine the answer. In rats (chapters 3 and 4) Musashi-1 protein levels in vivo were greater in Sertoli cell nuclei than cytoplasm in Sertoli cells which had ceased dividing and were undergoing differentiation.

In addition GATA-1 protein expression was shown to be restricted to non-proliferating Sertoli cells. It was hoped that in the present study the expression patterns of both Musashi-1 and GATA-1 could be investigated and differences between levels of expression in proliferating SK11 cells at 33°C and differentiating cells at 39°C could be assessed. GATA-1 mRNA has previously been detected in SK11 cells using RTPCR and preliminary investigations in this study also detected GATA-1 mRNA in SK11 cells using RTPCR techniques. However both mRNA and protein expression of GATA-1 could not be detected in the cultured cells using Northern blot analysis or immunocytochemistry. Moreover AR protein expression could not be localised to SK11 cells grown at either temperature using an antibody against AR. In previous studies AR mRNA was detected in the SK11 cell line using RTPCR. However it is possible that AR levels in the immortalised cell line are very low and could not be detected using immunocytochemical analysis. Levels of mRNA detected in immortalised Sertoli cell lines have been shown to be lower than in primary cultured Sertoli cells of the same species (McGuinness *et al.*, 1994). It has been proposed that the decrease in mRNA levels results from the expression of messages associated with cell replication which are not expressed in Sertoli cells which have generally ceased dividing when placed in vitro.

ERβ protein expression was detected in the nuclei of SK11 cells grown at both 33°C and 39°C. In agreement with the pattern of expression of ERβ protein in primary cultures of rat Sertoli cells (Chapter 5) ERβ was located to the nuclei of SK11 Sertoli cells cultured in the presence and absence of steroids. EB administration appeared to upregulate ERβ protein levels in SK11 cells cultured at 39°C. Levels of ERβ protein also appeared to increase in primary cultures of rat Sertoli cells following EB treatment (Chapter 5). In contrast to observed increases in AR protein levels following T administration thought to result from T stabilising the AR protein, Tena-Sempere et al., (2000) observed an increase in ERβ mRNA expression following in vivo EB treatment of prepubertal rat testis. Consequently it is proposed that in Sertoli cells EB directly upregulates ERβ protein expression at the level of gene transcription in addition to possibly stabilising the ERβ

protein. Results from the present study indicate that treatment with F+T also increases ERβ protein levels in SK11 cell nuclei even though AR expression could not be detected in SK11 cells. ERβ protein levels also appeared to be upregulated in primary cultured rat Sertoli cells (Chapter 5) and it was proposed that T indirectly increased ERβ levels through aromatisation of T into E. However previous research could only detect aromatase mRNA in SK11 cells cultured in the presence of F and not in the presence of F and T (Walther *et al.*, 1997). Therefore the mechanism by which T upregulates ERβ expression is unknown and requires further investigation. Additional information on the regulation of protein levels following steroid treatment will require Western analysis (Williams *et al.*, 2000).

Initially mRNA expression by SK11 cells was to be normalised against corresponding SGP-1 mRNA levels. However due to the findings in the previous chapter where SGP-1 mRNA expression appeared to be upregulated by T treatment, steroid regulation of SGP-1 expression in SK11 cells was also investigated. Administration of F, E and T downregulated SGP-1 mRNA expression in SK11 cells grown at both 33°C and 39°C and therefore mRNA levels were normalised against 18S rRNA levels. As SK11 cells are an immortalised cell line this was deemed appropriate as cultures were not contaminated by germ cells or peritubular cells which might have affected evaluation of Northern blots.

The transcription factor GATA-4 was expressed in nuclei of SK11 cells grown at both permissive and non-permissive temperatures. At 33°C GATA-4 mRNA and protein levels appear to be downregulated following 24 hour F treatment whereas the reverse situation occurred with GATA-4 protein levels in SK11 cells grown at 39°C. However results indicated that GATA-4 mRNA levels were downregulated in the presence of F at 39°C. It is possible that at 39°C F increases the stability of GATA-4 mRNA transcripts or increases the rate of GATA-4 translation in SK11 cells. Both these possibilities would result in an increase in GATA-4 protein without altering the number

of GATA-4 mRNA transcripts. There were inconsistencies between the two experiments regarding the effect of timed steroid treatment on SK11 cell GATA-4 mRNA expression. However overall it appeared that both E and T administration had little effect on GATA-4 gene expression in SK11 cells. This was in agreement with an absence of steroid regulation on GATA-4 protein levels in SK11 cells and similar results were obtained following steroid treatment of primary cultured rat Sertoli cells (Chapter 5) or in vivo treatment with DES on expression in day 18 rat testes (Chapter 4). Not all SK11 cells were immunopositive for GATA-4 and similar findings were also demonstrated in MSC-1 cultured Sertoli cells (Ketola *et al.*, 1999) and in primary cultures of rat Sertoli cells (Chapter 5). This is in contrast to GATA-4 protein expression in Sertoli cells observed in vivo where levels of expression appear constant across all tubules and at all stages of development (Chapter 3). The reason for the different levels of protein expression observed in cells in both immortalised and primary cultured Sertoli cells is unknown and requires additional research in order to establish the causes.

In the current study it was demonstrated that the immortalised mouse Sertoli cell line SK11, when grown at the non-permissive temperature, maintained expression patterns of genes and proteins characteristic of mouse and rat Sertoli cells in vivo and in vitro. ERβ protein levels appeared to be upregulated by EB and T treatment whereas GATA-4 protein levels were increased in the presence of F but were otherwise unchanged following steroid treatment. Disappointingly, expression levels of GATA-1 and Musashi-1 were too low in the immortalised cell line such that regulation of gene expression by steroids or Sertoli cell differentiation could not be determined. In order to address these questions future work employing RPA analysis would need to be carried out. Due to the maintenance of GATA-4 and ERβ protein expression in the SK11 cell line it is hoped that in future investigations possible interactions between GATA-4 and ERβ could be carried out. The presence of both proteins in the cell line and the

preservation of their normal in vivo regulatory processes demonstrates that these cells are good candidates in which to carry out co-immunoprecipitation experiments in order to determine if GATA transcription factors do interact with ERβ in the differentiating Sertoli cell.

Chapter 7.

General Discussion

The demonstration of ER expression throughout the male reproductive tract (Fisher *et al.*, 1997; Saunders *et al.*, 1998; van Pelt *et al.*, 1999) and the discovery that oestrogenic action was essential for fertility in the male mouse(Eddy *et al.*, 1996; Hess *et al.*, 1997) emphasised the need for further investigation into the role of oestrogen in the male reproductive system, in particular during postnatal testicular development. The hypothesis that increased levels of oestrogens and oestrogen like chemicals in the environment were causing detrimental effects on the male reproductive tract in particular during fetal and postnatal development (Sharpe and Skakkebaek, 1993) further highlighted the importance of establishing the complex mechanisms involved in the interplay between oestrogen and testis development. The aims of this study were to carry out investigations into the direct effects of oestrogen on postnatal Sertoli cell differentiation. Other studies had already shown that oestrogen treatment to pregnant rats could affect gene expression in the testes of their male fetuses (Majdic *et al.*, 1997; Majdic *et al.*, 1995). An alteration in the differentiation of Sertoli cells during postnatal development can affect the functional maturation of the entire testis (De Franca *et al.*, 1995). Changes in the expression patterns of established and novel markers of Sertoli cell maturation by oestrogen and testosterone were investigated using in vivo and in vitro methods and possible direct effects of oestrogen on Sertoli cell function were studied.

Expression patterns of novel markers of Sertoli cell functional maturation were investigated in Chapter 3. The RNA binding protein Musashi-1 was shown to be a prime candidate for a marker of Sertoli cell differentiation because the pattern of expression of Musashi-1 protein was modified as Sertoli cells switched from proliferating undifferentiated cells into morphologically and functionally differentiated cells. It is possible that Musashi-1 is involved in nuclear-cytoplasmic RNA trafficking in mitotic Sertoli cells and then becomes

predominantly involved in RNA processing within the nucleus during Sertoli cell differentiation. Modulation of Musashi-1 expression by steroids could not be demonstrated in this study using both in vivo and in vitro techniques which was disappointing. Unfortunately, expression of Musashi–1 appeared to be down regulated in primary cultures of immature rat Sertoli cells and mRNA levels were undetectable in the transformed mouse Sertoli cell line. Further research into regulation of Musashi-1 gene expression will require the use of more sensitive techniques such as real-time PCR or the use of alternative culture systems e.g. isolated seminiferous tubules (McKinnell and Sharpe, 1992; McLaren *et al.*, 1993). In addition investigations into which RNA transcripts bind to Musashi-1 in Sertoli cells could be carried out using immunoprecipitation and blot analysis although this would be technically challenging.

The transcription factors GATA-1 and GATA-4 bind to GATA motifs located in regulatory regions of a number of factors expressed in the postnatal testis (Feng *et al.*, 1998; Silverman *et al.*, 1999; Viger *et al.*, 1998). Therefore both GATA factors are thought to play an important role in testicular development. In this study GATA-4 protein expression was found to be restricted to Sertoli and Leydig cell nuclei throughout testis development. Expression patterns of GATA-4 in Sertoli cells, granulosa cells and cardiomyocytes reported in this study and in the published literature (Grepin *et al.*, 1995; Heikinheimo *et al.*, 1997; Ketola *et al.*, 1999), suggest that GATA-4 could act as a survival factor in these cell types. GATA-4 expression did not change during Sertoli cell maturation and the pattern of expression remained unchanged following delayed development in the neonatal oestrogenised rats (Chapter 4). GATA-4 was therefore not investigated further as a marker of Sertoli cell differentiation. Previous reports have suggested that expression of GATA-4 in Sertoli and granulosa cells is modulated by FSH and oestrogens respectively (Heikinheimo *et al.*, 1997; Ketola *et al.*, 1999) In this study GATA-4 mRNA and protein levels remained unchanged following treatment of isolated Sertoli cells with oestrogen, testosterone and forskolin. Research using more sensitive quantitative techniques will need to be carried out in the future to confirm these results. It would also be interesting to

study possible causes of non-uniform GATA-4 immunolocalisation in cultured Sertoli cell nuclei (see Figs 5.12 and 6.13) compared to the uniform GATA-4 protein expression observed in Sertoli cell nuclei present in intact rodent testis.

Over the course of this study, GATA-1 protein was shown to be an informative marker of Sertoli cell functional maturation as GATA-1 protein expression was restricted to non-proliferating Sertoli cell nuclei throughout postnatal testis development. Consistent with this, a delay in the cessation of Sertoli cell division induced in oestrogenised rat testis resulted in a delay in GATA-1 protein expression. GATA-1 expression was not observed in Sertoli cells until cell proliferation had stopped and the cells had begun to differentiate. Studies have shown that ERα and oestrogen complex with GATA-1 in erythroid cells (Blobel and Orkin, 1996; Blobel *et al.*, 1995). The ERα-ligand–GATA-1 complex prevents GATA-1 transactivation of genes essential for erythroid cell differentiation: consequently the erythroid cells enter the apoptopic pathway (Blobel and Orkin, 1996). It was hoped that during this study investigations into possible direct effects of oestrogen (in conjunction with ERβ) on GATA-1 transactivation of genes expressed in Sertoli cells could be carried out. If the described interactions in erythroid cells were also taking place in developing Sertoli cells such direct inhibition of GATA-1 transactivation by oestrogen and ERβ could result in a delay in Sertoli cell maturation. Disappointingly expression of GATA-1 mRNA in Sertoli cells could not be detected using Northern blot analysis or ribonuclease protection assays in either the in vivo or the in vitro studies. Preliminary RTPCR analysis was able to detect the presence of GATA-1 mRNA in differentiating Sertoli cells and Sertoli cells in culture. Additional investigations on the expression of GATA-1 will therefore need to make use of sensitive methods such as real-time PCR.

The amount of SGP-1 mRNA in total testicular extracts has often been used as an internal control to quantitate the amount of individual mRNAs in Sertoli cells in the presence of germ cells. It has been previously demonstrated that SGP-1 is constitutively expressed in Sertoli cells and that levels of expression do not

change across spermatogenic cycles, following hormonal treatment or during Sertoli cell development (Grima *et al.*, 1992; Mathur *et al.*, 1994; Sharpe *et al.*, 1993; Stallard and Griswold, 1990). However in the course of this study, SGP-1 mRNA and protein levels per Sertoli cell were shown to change as development progressed. SGP-1 levels were low in neonatal Sertoli cells but the amount of both SGP-1 mRNA and protein were upregulated as the Sertoli cells matured. It has also been stated that SGP-1 expression in the testis is restricted to Sertoli cell cytoplasm (Mathur *et al.*, 1994). However in the immature testes used in this study, SGP-1 protein was immunolocalised to macrophages and fetal type Leydig cells. These results highlight the need for further research into changes in SGP-1 expression during postnatal testis development. With more extensive research being carried out into the effects of oestrogen on prepubertal testis development, the use of SGP-1 as an internal control for Sertoli cell gene expression needs to be re-evaluated. It was notable that in vitro studies consistently demonstrated regulation of SGP-1 mRNA expression by testosterone in Sertoli cells which further emphasises the need to reassess the regulation of expression of SGP-1 in Sertoli cells. The majority of previous research into SGP-1 expression in the testis has been carried out on mature Sertoli cells. Work carried out during this study demonstrates additional differences between proliferating and differentiating Sertoli cells which need to be addressed when investigating Sertoli cell development in the future.

Expression of GATA-1, Musashi-1 and inhibin-α in cultured rat Sertoli cells and the transformed mouse Sertoli cell line, (SK11) appeared to be low/negligible demonstrating a problem which has been experienced during other studies using cell culture of Sertoli cells. Removal of a cell from its normal surroundings or transformation of the cell with a tumorigenic gene can lead to the loss of expression of certain genes (Angus *et al.*, 1993; Sutton *et al.*, 1998). However in this thesis isolation of Sertoli cells from intact rat testis was used as it provided a system in which the direct action of oestrogen and testosterone on Sertoli cell functional maturation could be explored without the complexities of the whole organism. The possibility of paracrine interactions within the testis masking the

regulation of the selected factors by oestrogen and testosterone was also removed using the in vitro culture systems. The studies described in this thesis were preliminary and therefore described effects of oestrogen on immature Sertoli cells which *could* take place during postnatal testis development. Due to the artificial nature of the experiments statements were not made proposing what *actually* does take place within differentiating Sertoli cells.

The primary rat Sertoli cell culture system employed in this study retained normal cellular physiological responses of Sertoli cells even after 5 days in culture in the absence of steroids. To the best of my knowledge these results are the first reports of immunolocalisation of ERβ protein to cultured Sertoli cells. Upregulation of ERβ expression in Sertoli cells following oestrogen administration supports previously published work where an upregulation in ERβ mRNA levels in neonatal and prepubertal rat testis following oestrogen administration was also demonstrated (Tena-Sempere *et al.*, 2000). These results suggest that oestrogen increases ERβ gene transcription in differentiating Sertoli cells leading to an increase in ERβ protein levels. This is in contrast to the proposed effect of testosterone on AR protein levels where testosterone stabilises the receptor rather than actually upregulating AR expression levels (Sar *et al.*, 1993). Such an upregulation of ERβ levels by oestrogen in immature and differentiating Sertoli cells provides a direct pathway by which exogenous oestrogens can affect Sertoli cell maturation and subsequent testis development. In addition preliminary results in this thesis supported previous reports of a downregulation in AR expression levels in differentiating Sertoli cells following oestrogen administration (Sharpe *et al.*, 1998; Tena-Sempere *et al.*, 2000). These observations provide an alternative pathway by which oestrogen can affect male development through inhibition of androgen action in Sertoli cells.

It must be noted that these results are from preliminary investigations and in most cases experiments were only performed twice, hence statistical analysis could not be carried out. This was partly due to the problem in obtaining sufficient numbers of Sertoli cells in the primary rat Sertoli cell cultures. Low cell

numbers restricted the quantities of RNA and protein that could be extracted from the cultures, which in turn limited the analysis of mRNA levels to the more abundant RNA transcripts. The importance of obtaining equal distribution of Sertoli cells across treatment groups and ensuring cell numbers were uniform across experimental groups was also demonstrated during the course of this study. It was hoped that the use of a transformed Sertoli cell line would overcome problems with variation between cultures however in the present study AR, GATA-1, Musashi-1 and inhibin-α expression levels were found to be very low and were below detection levels for immunocytochemistry and Northern blot analyses. Additional studies with these cells may therefore require transfection with reporter constructs to restore the expression of regulatory factors such as GATA-1.

In conclusion the studies described in this thesis have provided preliminary results regarding the effects and mechanisms of oestrogen on differentiating Sertoli cells. ERβ protein levels were upregulated by oestrogen administration and conversely AR levels were decreased with oestrogen treatment. Expression patterns of Musashi-1 and GATA-1 in developing testes demonstrated the potential use of these factors as markers of Sertoli cell functional maturation in future research. Novel results regarding the regulation and changes in levels of SGP-1 expression during Sertoli cell maturation were demonstrated. These findings strongly suggest that further research into SGP-1 expression in the developing testis is necessary if SGP-1 continues to be used as an internal control in studies on Sertoli cell gene expression. A number of problems associated with the use of in vitro systems were highlighted during these investigations including the need for sufficient cell numbers in primary Sertoli cell cultures, even distribution of cell numbers across treatment groups and loss of expression of some genes in cells removed from the normal environment within the seminiferous tubule. The overall conclusion from this work is that more effort is required to identify mechanisms of oestrogen action on Sertoli cells. This study demonstrated expression of ERβ in cultured Sertoli cells. In the future in vitro Sertoli cell culture could be utilised for further investigations into

the mechanisms of oestrogenic action on developing Sertoli cells. Future research could focus on the proposed interplay between oestrogen, ERβ and GATA factors within the developing Sertoli cell. This research could provide invaluable data necessary for the elucidation of the pathways involved in interactions between oestrogen and Sertoli cell maturation which ultimately affect male fertility.

Appendix: Some commonly used agars and buffers

Agars

Soc medium

20g Bacto-tryptone
5g Bacto-yeast extract
10mM NaCl
2.5mM KCl
10mM $MgCl_2$, in 1 litre

Luria Bertani (LB) agar plates

15g agar
10g Bacto-tryptone
5g Bacto-yeast extract
10g NaCl [pH 7.0], in 1 litre

LB broth

10g Bacto-tryptone
5g Bacto-yeast extract
10g NaCl [pH 7.0], in 1 litre

Buffers

1x TBE

89mM Tris base
89mM boric acid
10mM EDTA

TE buffer

10mM Tris-HCl
1mM EDTA
at pH8.0

1x SSC

0.15M sodium chloride
0.015M sodium citrate
at pH7.0

1x SSPE

0.15M sodium chloride
0.01M NaH2PO4, anhydrous
1mM EDTA
at pH7.4

1 x TAE

4mM Tris base
2mM sodium acetate
1mM EDTA
at pH 7.2

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